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# PARAMUTAGENIC ACTION OF PARAMUTANT $R^r$ AND $R^g$ ALLELES IN MAIZE[1]

DOUGLAS F. BROWN AND R. ALEXANDER BRINK

*Department of Genetics, University of Wisconsin, Madison, Wisconsin*

Received July 8, 1960

THE present article is concerned with an aspect of paramutation that was first noted during the study of the locus dependence of the paramutant $R^r$ phenotype (BRINK, BROWN, KERMICLE and WEYERS 1960). It was observed that, in $R^rR^{st}$ plants, the $R^r$ allele not only paramutated to a weakly pigmenting form but also it, in turn, acquired the capacity to promote paramutation. The change in $R$ expression which may be induced by such a paramutant allele ($R^{r'}$ or $R^{g'}$) is small relative to that regularly effected by $R^{st}$ in $R^rR^{st}$ individuals, and will be referred to as secondary paramutation.

## MATERIALS AND METHODS

The reader is referred to a previous paper (BRINK, BROWN, KERMICLE and WEYERS 1960) for a description of the materials and methods used in this investigation.

Measurement of the paramutagenic action of a paramutant allele requires that the paramutant and nonparamutant factors be distinguishable among the offspring of a heterozygote by a criterion independent of paramutation. This requirement is met in $R^{r'}R^g$ and $R^rR^{g'}$ plants by the contrasting seedling color effects of the $R^r$ (red) and $R^g$ (green) alleles.

The data which provide evidence for secondary paramutation were obtained from the following four testcrosses:

(1) $r^gr^g$ ♀ × $R^{r'}R^g$ ♂. The staminate parent in this cross was derived from the mating, $R^rR^{st}$ × stock $R^gr^g$.

(2) $r^gr^g$ ♀ × $R^rR^g$ ♂. The staminate parent used in this control mating was derived from the cross, stock $R^rR^r$ × stock $R^gr^g$.

These two testcrosses permit a test for paramutagenicity of $R^{r'}$. If secondary paramutation of $R^g$ occurs in $R^{r'}R^g$ plants, then the $R^gr^gr^g$ kernels resulting from testcross (1) should be more lightly pigmented than those from testcross (2).

(3) $r^gr^g$ ♀ × $R^{g'}R^r$ ♂. The staminate parent was the offspring of the mating, $R^gR^{st}$ × stock $R^rR^r$.

(4) $r^gr^g$ ♀ × stock $R^rR^r$ ♂.

Matings (3) and (4) permit a test for the paramutagenicity of $R^{g'}$ in $R^rR^{g'}$ plants. In this case, secondary paramutation would be evidenced by a reduced

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pigmentation of the $R^rr^gr^g$ kernels from testcross (3) as compared with the standard phenotype from testcross (4).

Testcrosses (1), (2) and (3) were carried out with each of eight $R^g$ mutants from the $R^r$ allele. Since these mutants and standard $R^r$ previously were found to be alike in both aleurone pigmenting capacity and paramutagenicity (BRINK, BROWN, KERMICLE and WEYERS 1960), the results of these crosses represent, in effect, eight independent tests for paramutagenicity of $R^{r\prime}$ and $R^{g\prime}$.

## EXPERIMENTAL RESULTS

The aleurone color scores of $R^gr^gr^g$ kernels from testcrosses (1) and (2) for each of the eight $R^g$ mutants are summarized in Table 1. The first line of each pair of frequency distributions contains the aleurone color data for kernels carrying the $R^g$ factor in question extracted from heterozygotes with standard $R^r$; the second line contains the comparable data for kernels carrying the corresponding $R^g$ allele extracted from heterozygotes with paramutant $R^{r\prime}$. The difference between the values for each pair of frequency distributions constitutes a measure of secondary paramutation in the heterozygotes containing $R^{r\prime}$. The $R^gr^gr^g$ kernels from test-

TABLE 1

*Frequency distributions and means of aleurone color scores for* $R^gr^gr^g$ *kernels on testcross ears from* $r^gr^g$ ♀ × $R^rR^g$ ♂ *(ex.* $R^rR^{st} \times R^gr^g$*) matings in comparison with those of* $R^gr^gr^g$ *kernels from* $r^gr^g$ ♀ × $R^rR^g$ ♂ *(ex.* $R^rR^r \times R^gr^g$*) control matings*

| Parentage of staminate testcross parent | No. of plants tested | Distribution of color scores for $R^gr^gr^g$ kernels only | | | | | | | Mean score for $R^gr^gr^g$ kernels |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | |
| $R^rR^r \times R^gr^g_1$ | 4 | .. | .. | .. | 5 | 84 | 77 | 2 | 5.45 |
| $R^rR^{st} \times R^gr^g_1$ | 10 | 1 | 8 | 9 | 251 | 123 | 28 | .. | 4.36 |
| $R^rR^r \times R^gr^g_3$ | 4 | .. | .. | .. | .. | 51 | 116 | 1 | 5.70 |
| $R^rR^{st} \times R^gr^g_3$ | 10 | 6 | 1 | 3 | 107 | 214 | 89 | .. | 4.88 |
| $R^rR^r \times R^gr^g_4$ | 4 | .. | .. | .. | 4 | 72 | 91 | 1 | 5.53 |
| $R^rR^{st} \times R^gr^g_4$ | 10 | 6 | .. | 3 | 104 | 249 | 57 | 1 | 4.82 |
| $R^rR^r \times R^gr^g_5$ | 4 | .. | .. | .. | 27 | 99 | 42 | .. | 5.09 |
| $R^rR^{st} \times R^gr^g_5$ | 10 | 8 | 8 | 33 | 266 | 91 | 14 | .. | 4.11 |
| $R^rR^r \times R^gr^g_6$ | 4 | .. | .. | .. | 6 | 81 | 81 | .. | 5.45 |
| $R^rR^{st} \times R^gr^g_6$ | 10 | 1 | 1 | 2 | 99 | 244 | 73 | .. | 4.91 |
| $R^rR^r \times R^gr^g_7$ | 4 | .. | .. | .. | .. | 80 | 87 | 1 | 5.53 |
| $R^rR^{st} \times R^gr^g_7$ | 10 | 1 | 1 | 4 | 129 | 225 | 60 | .. | 4.80 |
| $R^rR^r \times R^gr^g_8$ | 4 | .. | .. | .. | .. | 56 | 108 | 4 | 5.69 |
| $R^rR^{st} \times R^gr^g_8$ | 10 | 1 | 10 | 3 | 186 | 183 | 37 | .. | 4.55 |
| $R^rR^r \times R^g{}_{10}r^g$ | 4 | .. | .. | .. | 5 | 95 | 68 | .. | 5.38 |
| $R^rR^{st} \times R^g{}_{10}r^g$ | 10 | 2 | .. | 2 | 79 | 238 | 99 | .. | 5.02 |

crosses of $R^{r'}R^{g}$ plants are significantly lower in aleurone color score than those from testcrosses of $R^{r}R^{g}$ plants in each case. The average difference between the mean scores of the two classes is 0.80 of a class interval.

Table 2 contains the aleurone color data for $R^{r}r^{g}r^{g}$ kernels derived from testcrosses (3) and (4). The first line of the table shows the distribution of scores characteristic of standard $R^{r}$ in single dose. The remaining distributions in the table represent the aleurone phenotypes of kernels carrying $R^{r}$ genes extracted from heterozygotes with the eight respective $R^{g'}$ alleles. The difference between the mean score for control $R^{r}r^{g}r^{g}$ kernels from $R^{r}R^{r}$ staminate parents and the mean score for the $R^{r}r^{g}r^{g}$ kernels from each of the $R^{r}R^{g'}$ staminate parents is a measure of secondary paramutation in the respective $R^{r}R^{g'}$ heterozygotes. These differences, given in the last column of the table, are statistically significant in each case.

A direct comparison of magnitude of change in pigmenting capacity of the $R^{g}$ alleles induced by $R^{r'}$, as shown in Table 1, and that of $R^{r}$ induced by the $R^{g'}$ alleles, as summarized in Table 2, cannot be made because the two sets of data were not acquired in the same year and slightly different sets of standards were used in scoring the testcross kernels. These facts probably account for the seemingly greater change in $R$ expression due to secondary paramutation shown in Table 1 than in Table 2.

## DISCUSSION

The above results demonstrate that a standard $R^{r}$ or $R^{g}$ allele becomes weakened in pigmenting action when passed through a heterozygote carrying a paramutant $R$ factor. The change in pigmenting action of $R^{g}$ induced by $R^{r'}$ is small when compared to that induced by $R^{st}$. This relation is illustrated by the data in Table 3.

TABLE 2

*Frequency distributions and means of aleurone color scores for* $R^{r}r^{g}r^{g}$ *kernels on testcross ears from* $r^{g}r^{g}$ ♀ × $R^{r}R^{g'}$ ♂ *(ex.* $R^{g}R^{st}$ × $R^{r}R^{r}$*) matings in comparison with those of* $R^{r}r^{g}r^{g}$ *kernels from the control matings* $r^{g}r^{g}$ ♀ × *standard* $R^{r}R^{r}$ ♂

| Staminate testcross parent | No. of plants tested | Distribution of color scores for $R^{r}r^{g}r^{g}$ kernels only | | | | | | | Mean score for $R^{r}r^{g}r^{g}$ kernels | D† |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |
| $R^{r}R^{r}$ | 12 | .. | .. | .. | 2 | 62 | 437 | .. | 5.88 | ... |
| $R^{r}R^{g'}_{1}$ | 13 | 2 | .. | 1 | 25 | 156 | 404 | .. | 5.63 | 0.25* |
| $R^{r}R^{g'}_{3}$ | 15 | 2 | 3 | 4 | 60 | 240 | 321 | .. | 5.38 | 0.50* |
| $R^{r}R^{g'}_{4}$ | 14 | 2 | 2 | 1 | 40 | 195 | 348 | .. | 5.50 | 0.38* |
| $R^{r}R^{g'}_{5}$ | 15 | .. | 5 | 9 | 33 | 203 | 380 | .. | 5.50 | 0.38* |
| $R^{r}R^{g'}_{6}$ | 15 | 2 | .. | .. | 22 | 205 | 400 | 1 | 5.59 | 0.29* |
| $R^{r}R^{g'}_{7}$ | 15 | 3 | .. | .. | 26 | 187 | 413 | 1 | 5.60 | 0.28* |
| $R^{r}R^{g'}_{8}$ | 15 | 3 | .. | .. | 22 | 173 | 432 | .. | 5.63 | 0.25* |
| $R^{r}R^{g'}_{10}$ | 13 | 1 | 1 | 4 | 23 | 145 | 372 | .. | 5.61 | 0.27* |

* Significant at the .05 level of probability by Tukey's test.
† D = mean score for control $R^{r}r^{g}r^{g}$ kernels from $R^{r}R^{r}$ staminate parents minus the mean score for $R^{r}r^{g}r^{g}$ kernels from $R^{r}R^{g'}$ staminate parents.

TABLE 3

*Mean scores for* $R^gr^gr^g$ *kernels only from testcrosses of* $R^gR^r$, $R^gR^{r'}$ *and* $R^gR^{st}$ *plants*

| $R^g$ allele (1) | Staminate testcross parent | | |
|---|---|---|---|
| | $R^gR^r$ (2) | $R^gR^{r'}$ (3) | $R^gR^{st}$ (4) |
| $R^g_1$ | 5.45 | 4.36 | 2.20 |
| $R^g_3$ | 5.70 | 4.88 | 2.58 |
| $R^g_4$ | 5.53 | 4.82 | 3.07 |
| $R^g_5$ | 5.09 | 4.11 | 1.82 |
| $R^g_6$ | 5.45 | 4.91 | 2.66 |
| $R^g_7$ | 5.53 | 4.80 | 2.46 |
| $R^g_8$ | 5.69 | 4.55 | 2.20 |
| $R^g_{10}$ | 5.38 | 5.02 | 2.52 |

In Table 3, the results of three kinds of testcrosses are summarized. The figures in columns 2 and 3 have been brought forward from Table 1, and are the average aleurone color scores for $R^gr^gr^g$ kernels from testcrosses of $R^gR^r$ and $R^gR^{r'}$ plants, respectively. The scores in column 2 represent the level of pigmenting action of the $R^g$ alleles in standard form. Those in column 3 represent the level of pigmenting action of $R^g$ alleles that have been opposed to $R^{r'}$ and, therefore, have undergone secondary paramutation. The figures in the last column were obtained from results of $R^gR^{st}$ testcrosses which have been presented in a previous paper (BRINK, BROWN, KERMICLE and WEYERS 1960). The scores in this column represent the level of expression of the $R^g$ alleles which have undergone primary paramutation in heterozygotes with $R^{st}$. A comparison of the figures in columns 2, 3 and 4 of Table 3 for each $R^g$ allele shows that $R^{r'}$ is only weakly paramutagenic relative to $R^{st}$.

It is equally clear from the values in Table 2 that the change in pigmenting action of $R^r$ induced by the $R^{g'}$ alleles is also small relative to that induced by $R^{st}$.

## SUMMARY

Data are presented which show that paramutant $R^r$ and $R^g$ alleles derived from heterozygotes containing the $R^{st}$ factor are themselves weakly paramutagenic.

## ACKNOWLEDGMENTS

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## LITERATURE CITED

BRINK, R. A., D. F. BROWN, J. KERMICLE, and W. H. WEYERS, 1960 Locus dependence of the paramutant *R* phenotype in maize. Genetics **45**: 1297–1312.

# NATURAL SELECTION IN EXPERIMENTAL POPULATIONS OF TRIBOLIUM.

## I. PRELIMINARY EXPERIMENTS WITH POPULATION CAGES[1]

DANIEL J. McDONALD AND NANCY JANE PEER

*Department of Biology, Dickinson College, Carlisle, Pennsylvania*

Received March 24, 1960

THE development of the Drosophila population cage by L'HERTIER, NEEFS and TEISSIER (1937) has proved to be of considerable significance in the field of population genetics. For instance, with this technique they were able to observe the operation of natural selection in mixed mutant and wild type populations of *Drosophila melanogaster* and demonstrate in certain instances that alteration of the cage environment could completely reverse the course of selection.

Population cages have since been employed by numerous investigators studying adaptive differences between karyotypes found in wild populations of several Drosophila species. For example, DOBZHANSKY and his associates, making frequent use of the cage technique, clarified the role of heterosis in maintaining chromosomal polymorphism in Drosophila populations (see LEVENE and DOBZHANSKY 1958, for a brief review). The applicability of the cage technique to problems involving interspecific as well as intraspecific competition has been demonstrated by MOORE (1952a,b) who studied the ecological differences between *Drosophila simulans* and *Drosophila melanogaster* and noted an improvement in the competitive ability of *simulans*.

It is surprising that, despite the demonstrated utility of the population cage, there has been little interest in developing cages suitable for organisms other than the vinegar fly. The work of NICHOLSON (1957) with the sheep blowfly, *Lucilia cuprina*, seems to be the only instance of extensive effort in this direction. This lack of interest is unfortunate, for the examination of many different kinds of organisms will, by verifying observations already made and providing new information, lead to the formulation of general principles.

A series of experiments was planned, therefore, to explore the dynamics of genetic changes in populations of the flour beetles, *Tribolium confusum* and *castaneum*, reared in population cages, and where possible, to discover how such changes are linked to the population's ecology. This paper describes the design and maintenance of population cages for Tribolium and an attempt to estimate adaptive values of mutants in experimental populations of *Tribolium confusum*.

[1] This investigation was supported by research grants G-5488 and G-8888 from the National Science Foundation.

## METHODS AND RESULTS

*The population cages:* Adapting the basic design of fly cages to flour beetle cages must take into consideration two prime differences between Tribolium and Drosophila. First, the beetles rarely take to flight and for all practical purposes can be considered flightless. Second, all of the life stages of Tribolium inhabit the same medium—a dry flour and yeast mixture—whereas the embryonic stages of Drosophila occupy the medium, and the adults occupy the space above it. The second consideration, at least, has proved to be of some significance. A Tribolium population cage, therefore, need consist only of a container subdivided by removable compartments arranged immediately adjacent to each other. Two models with slightly different dimensions have been used, but the most convenient one consists of a one gallon stainless steel tank 13.0 cm wide, 18.5 cm long, and 18.0 cm deep, with a 1 cm overhanging rim projecting outward around the top. A strip of one-fourth inch foam rubber weather stripping is applied to this rim, and a plywood frame, with a piece of finely woven cloth stretched over it, is cut to fit over the top of the tank and the surrounding rim. Holes drilled through the rim and the frame are fitted with bolts and wing nuts so the frame can be securely fastened. Each cage is fitted with eight food compartments 4.5 cm wide, 6.5 cm long, and 3.3 cm deep, made of aluminum sheeting containing circular perforations 4 mm in diameter, spaced about 1 cm apart. A mixture of 95 percent sifted whole wheat flour and five percent Brewer's yeast is poured into the cage until the tops of the compartments are just visible. This quantity of medium weighs about 400 grams.

Following introduction of the beetles into a new cage, an undisturbed period of four to eight weeks is allowed before any medium changing takes place. Then, each week, either one or two compartments are replaced with fresh compartments and the adults, pupae, and larger larvae sifted from the old medium are returned to the cage. This medium changing schedule insures that a compartment remains in the cage for no less than six weeks or no more than seven weeks. It can be arranged so that two compartments are changed every fourth week when a gene frequency determination is made. In some earlier experiments, where a cage with 15 smaller compartments was used, this schedule was modified, but the over-all result has not seemed to be affected by this. All population cages and other experiments described below were incubated at 30±1°C and a R.H. of 50–65 percent.

Although several mutations of *Tribolium confusum* and *Tribolium castaneum* have been studied, this report will be concerned only with the mutations Striped (*St*) and black (*b*) of *Tribolium confusum*.

*The Striped mutation:* Striped (McDonald 1958, 1959) is a sex-linked lethal which brings about the destruction of almost all hemizygous males ($St\,\delta$) and reduces the viability of heterozygous females ($+/St\,♀$) (Table 1). Surviving mutant males are usually sterile, live only a few days, and have solid white elytra. Heterozygous females (hereafter designated *St* females) possess two white

TABLE 1

*Offspring from crosses of heterozygous females* (St *females*) × *wild type males*

| Phenotype<br>Genotype | wild ♀<br>+/+ ♀ | *St* ♀<br>+/*St* ♀ | wild ♂<br>+ ♂ | mutant ♂<br>*St* ♂ |
|---|---|---|---|---|
| Totals | 1,971 | 1,563 | 1,618 | 68 |
| Percent | 37.7 | 29.9 | 31.0 | 1.3 |

stripes running the lengths of the elytra, permitting rapid separation from wild type females.

The results of several experiments (Figure 1) indicate that the lethal gene is effective during the egg stage and the first few days of larval life. In these experiments, 50 vials were prepared, each containing three grams of fine flour medium and 40 eggs, not more than 12 hours old, collected from *St* females mated with wild males. A control was set up with eggs from wild females. At intervals the contents of each vial were passed through a fine sieve and the number of living and dead larvae recorded. The data plotted in Figure 1 reveal that six days after the eggs have been deposited there is an average of 29.06 living larvae per vial in the experimental series and 31.96 per vial in the control. Since not more than three dead larvae were found in the entire series of experimental vials at this time, the deficit of living individuals at the sixth day may have been caused by deaths occurring prior to hatching. Obviously, by the 12th day most of the lethality due to the *St* mutation has taken place, for the numbers which have died by this time are enough to account for the shortage of mutant males and females at eclosion. Some wild type males may perish during this period too, but this cannot be determined without further refining the experiment.

Because of the early death of most mutant males and the sterility of those surviving, selection against them is probably complete. The mathematical model for complete selection against a recessive sex-linked mutation (HALDANE 1927) demonstrates that the frequency of heterozygous females in the female segment

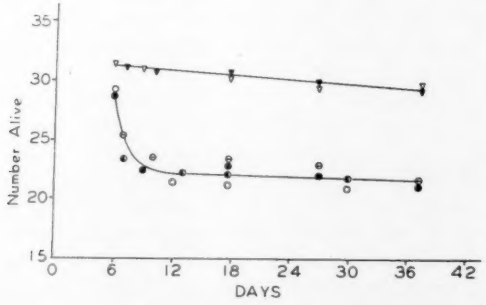


FIGURE 1.—The mean number of larvae alive per vial at different ages. Upper curve: larvae from wild female × wild male crosses (two experiments). Lower curve: larvae from *St* female × wild male crosses (four experiments). The different symbols are for individual experiments.

of the population will decrease each generation to one half its value in the previous generation. However, since the model will need modification if the heterozygous and wild type females have different adaptive values, this aspect of the problem must receive further attention.

Two major factors which could make the adaptive values of the females different are preadult viability and development rate. SPIESS (1958), investigating these and other life cycle components which might influence adaptive values in populations of *Drosophila persimilis*, found that the adaptive values of the karyotypes could be accounted for on the basis of differences in preadult viability and rate of development.

In our material it is clear that under certain conditions the preadult viability of *St* females is only about 80 percent that of the wild type (Table 1), so that the frequency of *St* females will be reduced each generation to four tenths of its previous value rather than five tenths. Unfortunately, there are no data available, at this moment, showing the effects of crowding or other environmental variables on preadult viability. However, to test the validity of the reasoning involved thus far, a relatively simple situation was examined where complications, introduced by crowding and medium conditioning, were less likely to be felt.

In this experiment, a population was reared in single discrete generations and the frequency of *St* females in the female segment of the population was measured each generation. The initial population consisted of 1000 adult females and 500 males all about a week old. The frequency of *St* females was 0.95 and of wild type females was 0.05. This population was introduced into a one gallon mason jar with about 400 grams of medium, allowed to remain there for three weeks, and then removed and discarded. About 1100 pupae were collected and sexed over the next three-week period. After eclosion, the females were classified as *St* or wild and counted. The entire sample was then introduced into a new jar of medium and the previous routine repeated. A second identical experiment was run simultaneously. Figure 2 demonstrates clearly that with these conditions the decline in frequency of *St* is largely the result of its lethal effects on mutant males and *St* females, for the frequencies obtained each generation are quite close to those predicted for an adaptive value of 0.4.

A further examination of the *St* strain revealed a small difference between the development rates of *St* and wild females. The results given in Figure 3 were obtained from experiments started by allowing *St* females mated with wild males to deposit eggs for 24 hours in a pint mason jar containing 135 grams of fine medium. Female pupae were collected as they appeared, and collections were continued until all the larvae had pupated. The collected female pupae were examined daily during the eclosion period, and the adults appearing each day were classified as *St* or wild. As Figure 3 shows, the proportion of wild females was significantly greater (chi-square = 18.55, $P<.01$) among those eclosing first, indicating a more rapid rate of development for them. The data suggest a rate difference of not more than a day, for the mean development time is about 29.7 days for the wild females and 30.1 days for the *St* females. However, since the average adult female may live five to six months (PEARL, PARK and MINER 1941)

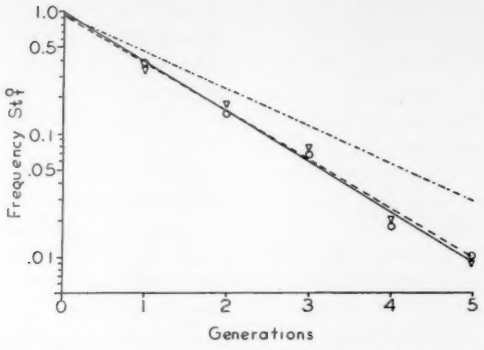


FIGURE 2.—The mean frequency per generation (solid line) of *St* females in the female segment of the population (two experiments averaged together) and the predicted frequencies per generation where *St* females have adaptive values of 0.4 (dashed line) and 0.5 (dot-dash line). The symbols ○ and △ indicate the observed frequencies of heterozygous females in the two experiments.

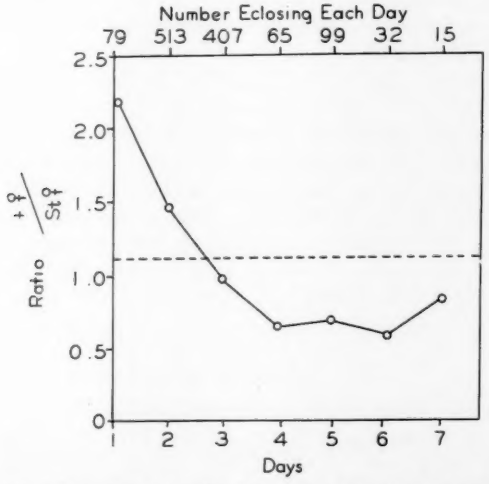


FIGURE 3.—Solid line: the ratio of $+\,♀/St\,♀$ among total females (indicated at top of figure) eclosing each day. A few eclosing after the seventh day were not included. Broken line: the mean ratio for the seven-day period.

and is productive for more than two months, this difference may have little effect in determining adaptive values.

A third factor which has received preliminary study is the longevity of the two kinds of females. Census data from the population cages (to be presented later) indicate that, in Tribolium population cages, longevity must be considered in

estimating generation time. Since the calculation of adaptive values requires some estimate of generation time, longevity, under varying conditions, must be carefully studied. PEARL *et al.* (1941) found the mean life span of a wild strain of *Tribolium confusum* to be 177.8 days for the males and 198.5 days for the females. The conditions were probably quite favorable, for in each vial there were only 16 beetles in 32 grams of flour which was renewed every five days. Further, since the sexes were separate, no sexual demands were made upon the insects. The results of the longevity experiment reported here are given in Figure 4. Seven vials were prepared, each containing five grams of medium and 50 adults—40 *St* females and ten wild males all within 24 hours of the same age. A second set of vials, containing the same amount of medium, had half as many adults of each kind. Two sets of control vials with wild type, instead of *St*, females were examined simultaneously. The data in Figure 4 suggest that under more crowded conditions the life span of *St* females is shorter than that of wild type females. Census data, to be discussed later, indicate that the density of adults in the population cages fluctuates between a high of about 25 per gram to a low of less than one, so that the adaptive value of *St* females may vary to the extent that it depends upon longevity. Since the vial environment differs in several respects from the cage environment (for example, the medium in the cages is older and usually contains more larvae and pupae), it probably would be unwise to draw further conclusions at this time.

*The black mutation:* The black strain of *Tribolium confusum* used in these experiments was generously supplied by DR. J. STANLEY, who in collaboration with H. M. SLATIS determined the inheritance of this mutation and compared the mutant development rate with that of wild type strains in the autotrephon, a device which samples a population automatically at set intervals (STANLEY and SLATIS 1955). Black (*b*) is inherited as an autosomal single gene difference. Since

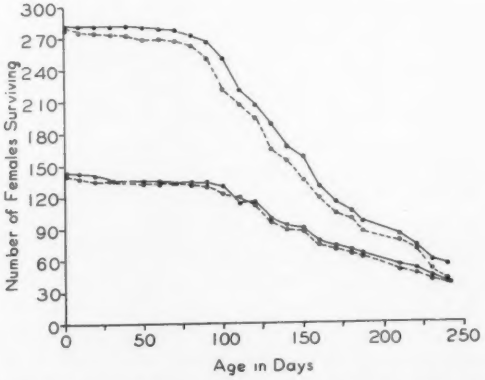


FIGURE 4.—The number of surviving *St* females (broken line) and wild females (solid line) at densities of ten beetles per gram of medium (upper curves) and five beetles per gram (lower curves).

dominance is absent, the heterozygotes are a deep reddish-black color and can be distinguished from the solid black homozygous mutant and the lighter red wild type. This makes it possible to utilize the method devised by LEVENE (DOBZHANSKY and LEVENE 1951) for the estimation of the adaptive values of the three different genotypes in a population. When reared in the autotrephon, the black strain requires 29.3 days for development from egg to adult as compared to 28.5 days for wild type strains. This fact might be expected to lower the adaptive value of the black homozygotes and, in the absence of any other differences, bring about a decline in frequency of the *b* gene in a population.

While no effects of the *b* mutation on fecundity, preadult viability, and other adaptive characteristics have been clearly established, their existence is not unlikely. For example, PARK, GINSBURG and HOROWITZ (1945) have demonstrated that the fecundity of the ebony mutant of *Tribolium confusum* is ten percent lower than the wild strain, while egg hatchability and development rate are not measurably different from the wild type. Studies of these and other adaptively significant traits in the black strain have recently been started.

*The Striped populations:* The only previous information on the subject of selection in populations of mutant and wild strains of Tribolium is contained in a paper by KOLLROS (1944). In these experiments, populations of *Tribolium castaneum* containing the pearl mutation and its wild type allele were maintained by transferring all the living forms to a fresh jar of medium every 30 days, at which time the mutant gene frequency was determined and a count made of larvae, pupae, and adults. The absence of any obvious trend in the gene frequency fluctuations of the various populations examined made it difficult to draw conclusions but, in general, selection seemed to proceed against the mutant.

In view of the deleterious effects of *St*, there was little doubt that it would be eliminated rapidly from the continuous populations reared in population cages. The question was whether the decline in *St* female frequency would be a constant logarithmic function of generations as it proved to be in the single generation experiments described earlier. This relationship implied that the adaptive values of the various genotypes were constant and independent of the change in the frequency of *St*. If this same relationship continues to exist in the population cages, and the passage of generations (population turnover) is a continuous process and occurs at a constant rate, then the decline in the frequency of *St* females should be a logarithmic function of time.

Two cages, numbered 10 and 11, were started, each containing initially 452 *St* female adults, 24 wild female adults, and 24 wild male adults, all about two-weeks old. The cages were undisturbed for six weeks, and then each week one or two compartments were replaced as described in the preceding section. At each renewal time the pupae, live adults, and dead adults sifted out of the old medium were counted and the living forms returned to the cage; every fourth week the frequency of *St* females among all the adult females in the sample was determined. The decline in the frequency of *St* females can be seen in Figure 5. In cage 10, the frequency dropped abruptly from the original 0.95 to 0.38 at the ninth week and seemed to decline gradually to 0.132 by the 37th week. Here, unfortunately,

cage 10 was accidentally discontinued. Cage 11 followed a similar course and then declined abruptly again following the 37th week. The absence of linearity in the plotted data of Figure 5 indicates that the decline in the frequency of *St* is not a constant logarithmic function of time. That this is due largely to a lack of continuity in population turnover is suggested by the following analysis of the census data.

In Figure 6, the numbers of live adults, dead adults, and pupae sifted from a compartment of old medium each week are plotted. From these compartment samples an attempt has been made to estimate the total number of live adults in

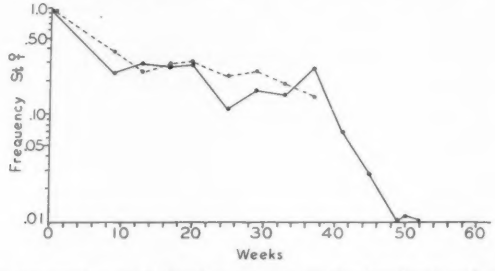


FIGURE 5.—The frequencies of *St* females in cage 10 (broken line) and cage 11 (solid line) determined from samples of about 200 females from each cage.

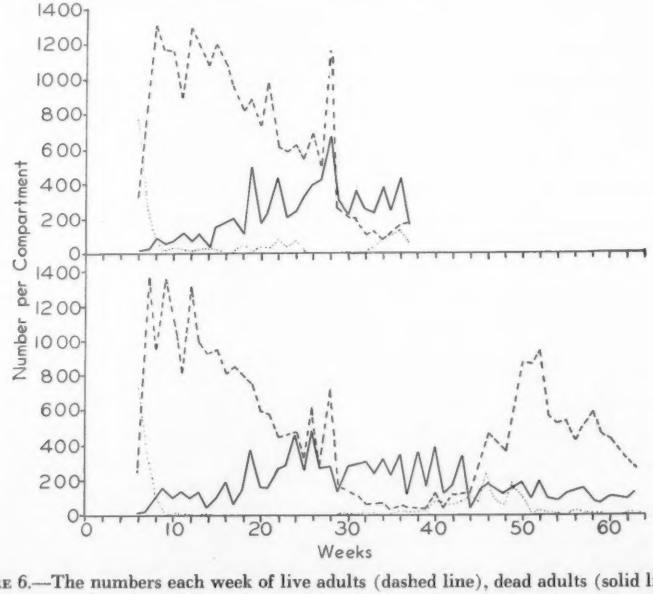


FIGURE 6.—The numbers each week of live adults (dashed line), dead adults (solid line), and pupae (dotted line) per compartment in the *St* population cages number 10 (upper curves) and number 11 (lower curves).

the cage each week, the total number of new adults added by eclosion from the pupae supply (births-per-week), and the total number of adults lost through death (deaths-per-week). To accomplish this, it was necessary to make certain assumptions concerning the population.

The assumptions are the following: (1) that live adults, dead adults and pupae are distributed uniformly throughout the cage; (2) that every dead adult is eventually recovered and counted; (3) that every pupa, after it has been counted and returned to the cage, eventually becomes an adult. Some doubt is cast on the validity of the first of these by the work of NEYMAN, PARK and SCOTT (1955), who report that adults tend to concentrate in the corners of a rectangular container filled with flour. However, this may not be a critical factor in population cages since every compartment has corners of its own regardless of its position in the cage. The distribution of dead adults and pupae probably depends upon the distribution of live adults and larger larvae, respectively. Preliminary observations indicate that departure from a random distribution of these forms is not very great.

The second assumption ignores the possibility that the dead adults disintegrate or are destroyed by the living insects. However, if these were common occurrences, it is likely that fragments of dead adults would be abundant in the old medium, whereas such debris is very infrequently encountered. Finally, in view of the cannibalistic propensities of Tribolium, the last assumption is at least questionable, but the rarity of partially consumed pupae suggests that pupae are not extensively cannibalized under the present conditions.

Despite the questionable validity of these assumptions, the following calculations which proceed from them indicate that they are not entirely unrealistic. If the live adults are distributed approximately at random throughout the cage, multiplying the live adult sample by the number of compartments (eight in this case) provides an estimate of the total live adults for that week. By the same reasoning, if all compartments have about the same number of pupae, multiplying the pupal sample by eight produces an estimate of the total pupal population. Two determinations of the time required for pupae to develop into adults under comparable temperature and humidity conditions are given by STANLEY (1946) and STANLEY and SLATIS (1955) as 6.67 and 6.19 days. If an intermediate value of 6.5 days is chosen, this would mean that the total pupal population at the time of sampling should have eclosed 6.5 days later. The number eclosing in seven days would be a trifle more than the total and can be obtained by multiplying the total pupae by 1.076. Thus, the births-per-week during the week following a sample may be determined directly from the pupal sample by multiplying it by 8.6.

The deaths-per-week seemed simple to determine, for if the dead adult sample is representative of the numbers which have died in all the other compartments over the preceding six weeks, deaths-per-week would be obtained by multiplying the dead adult sample by 8/6 or 1.33. Since the deaths-per-week figure is always calculated from the number dying over a six-week period, the figure is assigned to the approximate midpoint of each period, that is, the fourth week.

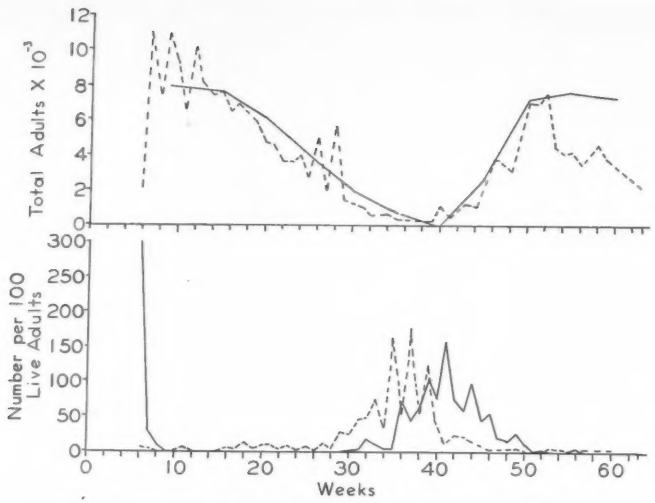


FIGURE 7.—Upper curves: the live adult total in cage 11 determined from the census sample (broken line) and the live adult total calculated from births- and deaths-per-week (solid line). Lower curves: the birth rate (solid line) and death rate (broken line) in cage 11.

In view of the number of assumptions involved in the calculation of births- and deaths-per-week, and the total live adults, it seemed desirable to verify the results obtained. A possible method presented itself with the realization that if the total number of living adults present at the time of a weekly census was increased by the births-per-week and decreased by the deaths-per-week, the resulting figure should be the number of living adults present in the cage at the next weekly census. The upper curve in Figure 7, obtained in this way, reveals that the total live adults calculated from births and deaths is comparable to the actual total calculated from the live adult sample, indicating that the assumptions involved in the census calculations cannot be too unrealistic. The greatest divergence, starting at about the 50th week where the derived total is higher than the actual total, might be due to overestimating births-per-week or underestimating deaths-per-week. Both these eventualities would follow if the active insects destroyed significant numbers of pupae and dead adults.

In a closed population such as this, an adult can enter the population only through birth and leave only through death, and unless either one or both of these is happening, there will be no change in the frequency of genes among the adults. The greatest changes in gene frequency can be expected, then, only when the birth rate, death rate, or both are relatively high. Where selection operates against a genotype through lowering preadult survival, a decline in this genotype will occur when the birth rate is high. In the population of cage 11, the birth rate, defined here as the number of pupae hatching per week per 100 live adults and plotted in Figure 7 (lower curve), was quite high when first measured on the

sixth week and undoubtedly had been high since the fifth week, when the first generation of adults must have begun to emerge. By the ninth week, when the maximum number of approximately 10,000 adults was present, the frequency of *St* females among all females had declined from the original 0.95 to 0.22. It seems clear that the major part of this decline was caused by the effect of the *St* mutation on preadult survival during a period of high birth rate, for the low adult death rate—that is the number of adults dying per week per 100 live adults —at this time precludes the possibility that the decline was due to a longevity difference between *St* and wild females. An essentially similar situation prevailed in cage 10 where the *St* female frequency of 0.38 coincides with the frequency found in the single generation experiments described earlier. In both cages, the birth rate had now reached a near minimum value and remained low in cage 11 until the end of the 35th week, probably because of egg cannibalism and a reduction in fecundity as the medium became conditioned with time (PARK 1933, 1938). Up to this point, the populations had behaved similarly to those studied by PARK (1948) who observed violent fluctuations in the numbers of live adults and immature stages early in the populations' histories. However, his *T. confusum* populations usually attained a condition of relative stability which persisted until the experiments were terminated. Further consideration of the data presented here indicates that this condition does not ensue in population cages.

Figure 7 shows that the death rate remained low up to the 18th week, when a noticeable increase set in, attained a maximum on about the 37th week, and declined sharply beyond the 40th week. Since most of the females in the cage up to this time were of the first generation and probably emerged around the seventh week, the increase in death rate on the eighth week indicates that death begins to strike when the adults are 11 weeks of age. This concurs with the data from the longevity experiment where the number of living insects declines steadily beginning with the 13th week. The relative stability in the *St* female frequency between the ninth and 37th week coincides approximately with the period of gradually increasing death rate and almost negligible birth rate, lending further support to the inference that a mutation such as *St*, whose principal action is to depress preadult survival, will not undergo a rapid change in its frequency when birth rate is low. The gradual decline which may have occurred can be attributed to such factors as a slightly shorter life span for *St* adults and the emergence of a few second generation individuals. The lower density of adults and consequent reduction in cannibalism are probably the cause of the increase in birth rate starting at about the 35th week, which was accompanied again by a sharp decline in the *St* female frequency that virtually eliminated *St* from the population. Thus, the census data provide an insight into the selection mechanism which would not be obtained by observing gene frequency changes alone and support the hypothesis that the stepwise decline in the frequency of *St* females was caused by the periodicity of population turnover.

If the decline in birth rate during the seventh week indicates almost complete emergence of the first generation, and the maximum death rate of the 37th week marks the approximate end of this generation, then the first generation endured

about 30 weeks. Unfortunately, there is reason to believe that the generation time will not be the same in subsequent generations, nor will each new peak. if the population continues to oscillate, indicate the emergence of a new generation. It is clear in cage 11 that, following the 35th week and even for a few weeks prior to it, new adults have been emerging for the birth rate begins to climb again at this time. However, unlike the situation at the beginning of the first generation, the birth rate now continued to increase for the next six weeks and was still substantial eight weeks after this maximum rate was reached. Thus, new individuals had been entering the population for over 16 weeks. Undoubtedly, second generation adults, emerging after the 35th week, mate and produce eggs which survive and about five weeks later emerge as third generation adults. Since these second generation adults are young and fertile, and the density of adults is relatively low, there is reason to believe that a large proportion of adults emerging after the 40th week are of the third generation, and that some emerging after the 45th week are fourth generation. Thus, the new population of adults, which reached its maximum size of about 7500 on the 52nd week, was a mixture of second, third, and fourth generation individuals. This may account for the decline in the *St* female frequency to a value far below that predicted if the new population had been second generation only.

From these studies, a pattern seems to be emerging which suggests that a continuous population of *Tribolium confusum* in population cages passes through two alternating phases. One phase is characterized by a low birth rate and a gradual decline in adult numbers from a maximum to a minimum value. Changes in gene frequency occurring during this phase would be largely the result of differential death rate. The second phase involves a high birth rate and a resultant increase in adult density to its maximum value. In this phase, a differential birth rate, whatever its cause, would be responsible for gene frequency changes occurring during the expansion of the adult population. Figure 7 indicates that this phase ended in cage 11 on the 51st week, when the birth rate dropped to a negligible value and the adult density reached a new peak. The complete cycle from the first adult peak to the second occupied about 43 weeks, and if two or three generations occur during each cycle, then the generation time would be about 14 to 21.5 weeks. Whether the population will continue to fluctuate in this way and what the duration of each phase will be are questions for the future. However, some data pertinent to these questions are available from the population cage experiments involving the black mutant.

*The black populations:* Two cages containing, originally, a mixture of black and wild type *T. confusum* in equal proportions have been maintained continuously for about two and one half years. Since in these earlier cages a considerable amount of medium is not confined to the removable compartments, a factor of uncertainty is introduced into the census calculations. These experiments were originally intended to provide information on genetic changes only, so census data were not obtained from them during the first 73 weeks of operation. However, Figure 8, which shows the numbers of live adults, dead adults, and pupae per compartment, demonstrates that the populations have not reached a stabile condition

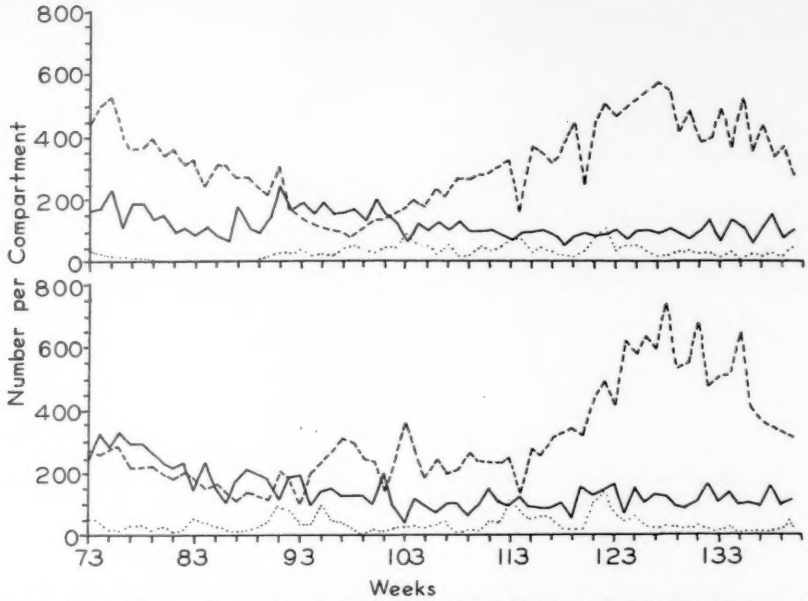


FIGURE 8.—The numbers each week of live adults (dashed line), dead adults (solid line), and pupae (dotted line) per compartment in the black population cages number 1 (upper curves) and number 2 (lower curves).

although they had been in existence for 73 weeks at the time of the first census. Cyclical changes are perhaps most obvious in the live adult totals, particularly in cage 1, where a complete cycle seems to have occurred during a one year period. Unfortunately, it is again not possible to determine how many generations have passed during this period. However, the relative abundance of pupae from the 89th to the 133rd week indicates that new generations were entering the population during all of this time. If five weeks is taken as the time required for a female to develop from an egg and attain sexual maturity, then the new adult population in cage 1, reaching its peak on the 127th week, may have contained individuals from eight or nine consecutive generations. It seems likely, however, that the greater proportion of them would be of the first few generations emerging, for as the density of adults increases, the probability of survival for eggs and pupae decreases. Therefore, if it is assumed that about three generations occurred during this cycle and the cycle required about 52 weeks, the generation time would be approximately 17 weeks.

A method has been devised (DOBZHANSKY and LEVENE 1951) for estimating the adaptive values of the three genotypes found in a population where two alleles with incomplete dominance are segregating. It should not be necessary to reproduce here the method of calculation, since excellent examples are available in the original report and in papers by SPIESS (1957) and HOCHMAN (1958). The geno-

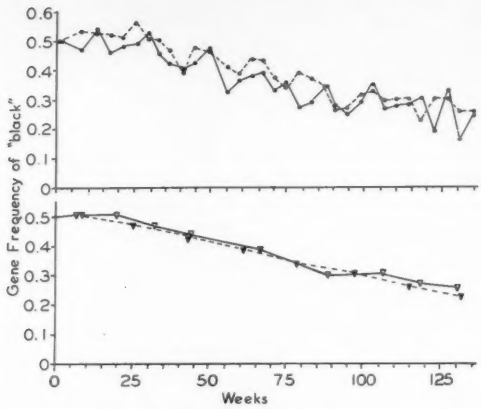


FIGURE 9.—Upper curves: the frequencies of the gene "black" in two population cages determined approximately every fourth week from a sample of 200 adults per cage. Lower curves: the average gene frequency of "black" in the two cages (solid line and △) and the theoretical selection curve (broken line and ▲) based on calculated adaptive values.

typic frequencies determined from a monthly sample have been used to calculate the frequencies of the *b* gene plotted in the upper curve of Figure 9. Clearly, the trends of the curves are downward, with a suggestion of a plateau at the start. The fluctuations are eliminated in the lower curve of Figure 9 by averaging together the gene frequencies of both cages and every three successive samples. From the pooled frequencies, using a generation time of 18 weeks, adaptive values of 0.95 for *bb* homozygotes and 1.30 for wild type homozygotes were calculated, assuming the *b*/+ heterozygotes have a value of 1.00. With these values, a satisfactory theoretical curve was constructed as shown in Figure 9. For the moment, these calculations are tentative until better estimates of the generation time become available. In fact, if further observations of these populations indicate that population turnover is continuous rather than periodic, reconsideration of this entire question may become necessary.

## DISCUSSION

It is now clear that populations of *Tribolium confusum* can be maintained for extended periods in population cages similar in design to those described here. Furthermore, some of the inherent advantages and disadvantages of using Tribolium for population studies of this kind have become apparent.

The principal disadvantage arises from the fact that a Tribolium population reproduces more slowly than, for example, a Drosophila population. SPIESS (1957) estimates the length of a generation of *Drosophila persimilis* in population cages at 15°C as seven weeks, while HOCHMAN (1958) used a generation time of about two weeks for *Drosophila melanogaster* at 25°C. The estimates of generation time

for *Tribolium confusum*, which at the moment lie on very uncertain ground, range from 14 to 21 weeks at 30°C. Despite the uncertainty, it seems unlikely that the figure for *Tribolium confusum* approaches seven weeks, and it cannot possibly be two weeks. However, since PARK (1954) has found that *Tribolium confusum* will readily tolerate a temperature of 34°C, the feasibility of hastening population turnover by using a higher incubation temperature is under consideration. Experiments are also planned to provide a more reliable estimate of the length of a generation in the population cage by following the oscillatory changes in the frequency of a sex-linked gene with initially different frequencies among males and females.

The greatest advantage of Tribolium populations seems to lie in the ease with which information about the size and age structure of the population, as well as genetic data, can be obtained. This fact should make Tribolium a particularly useful organism for studies of the relationships between the genetical and ecological aspects of populations (see BIRCH 1960, for a review of this subject).

The absence of obvious long-range oscillations in the numbers of adults and immature stages in the *T. confusum* populations studied by PARK (1948) has already been noted. The evidence presented here indicates that in population cages, live adult numbers oscillate for some time after the cage is started and may continue to do so indefinitely. At the moment, it is not clear why there should be this difference. In some later experiments, PARK (1954) found oscillation occurring in populations of both *T. confusum* and *castaneum*, probably in response to seasonal changes in relative humidity. Since the live adult cycle of cage 1 (Figure 8) approximates a year in length and is consequently in phase with the seasons (the high points occurred in November, 1958 and December, 1959), this explanation may prove to be applicable here. NICHOLSON (1957) demonstrated that oscillation in blowfly populations could be damped by adjusting the food supply of adults and larvae so that the effects of adult and larval competition would be in balance. It is at least possible that the frequency with which food supply is renewed and waste products removed might influence the conditions of equilibrium attained by Tribolium populations, so that under one set of conditions the population might be stabile, and under another it might oscillate. Unfortunately, the experiments described here were not designed to explore this problem.

Several new population cages are now yielding information which will help to establish how much truth is contained in the assumptions employed in the calculations of mortality and natality. A census of all the compartments simultaneously should reveal the true pattern of distribution of insects, living and dead, which is now assumed to be random. Other experiments will measure the loss of pupae and dead adults resulting from the activities of the living individuals.

Finally, the problem of the adaptive inferiority of homozygous and heterozygous black individuals remains to be clarified. It will be interesting to see if the slower development rate of the black strain (STANLEY and SLATIS 1955) is sufficient to account for its behavior in the population cages, or whether other causes must be sought.

SUMMARY

The structural details of population cages for the flour beetle, *Tribolium confusum*, and procedures for maintaining continuous populations of these organisms are described. Results of experiments exploring the relations between the genetical and ecological structure of *Tribolium confusum* populations are considered. The adaptive value of the Striped genotype, estimated from longevity and preadult viability data, was used to predict how rapidly the frequency of the *St* gene, a sex-linked lethal, would diminish in a population. Analysis of data collected from populations of *Tribolium confusum* reared in the population cages revealed that mortality and natality information could be used to interpret more adequately the changes in the frequency of Striped.

Gene frequency data collected from other cages containing the autosomal gene black and its wild type allele were used to calculate the adaptive values of the three genotypes. The values arrived at were: +/+, 1.30; +/*b*, 1.00; *b*/*b*, 0.95.

ACKNOWLEDGMENTS

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# THE GENETICS OF THE H SEROTYPE SYSTEM IN VARIETY 1 OF TETRAHYMENA PYRIFORMIS

D. L. NANNEY[1] AND J. M. DUBERT[2]

*Biology Division, California Institute of Technology and Zoology Department, University of Illinois*

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PREVIOUS studies on serotypes of variety 1, *Tetrahymena pyriformis*, demonstrated that a single strain grown under different conditions could manifest any of several different serotypes even though any one cell normally expressed only one serotype at any one time (LOEFER, OWEN and CHRISTENSEN 1958; MARGOLIN, LOEFER and OWEN 1959; INOKI and MATSUSHIRO 1958). Strains of independent origin, moreover, manifest different arrays of serotypes and can often be distinguished by their responses to antisera. In particular, the available strains of variety 1 can now be classified into five distinct categories when grown at 25°C in an Aerobacter-Cerophyl medium (LOEFER and OWEN, in press). These serotypes are designated as the H serotypes, specifically, Ha, Hb, Hc, Hd and He. This report is concerned in part with the genetic basis for the strain differences in H antigens.

The H serotype system is also characterized by "vegetative instability" of the phenotypes in the progeny of certain crosses. Preliminary analysis of the intraclonal variation within the serotype system suggests that a novel form of nuclear differentiation is involved.

## MATERIALS AND METHODS

The Ha serotype was first identified in the wild strain WH-6 and is now established in an inbred strain designated as Family A. Hb was detected in strain WH-52; it has not been established in an inbred series, and the prospects for doing so are meager since WH-52 is now virtually sterile. Hc first appeared in an early generation of an inbred series called Family B; it is absent from the terminal members of the same series, but has been established in another inbred line called Family D1. Serotype Hd was originally detected in a wild strain, WH-14, and is now available in the inbred series designated as A1, B and D. Finally, He was discovered in strain UM-226 and is established in inbred series C. These serotype designations differ from those in the original publication (MARGOLIN, LOEFER

[1] Present address: Zoology Department, University of Illinois, Urbana. The support of the National Science Foundation through grants and a Senior Postdoctoral Fellowship is gratefully acknowledged.

[2] Permanent address: Service de Biochimie Cellulaire, Institut Pasteur, Paris. Appreciation is expressed to the Rockefeller Foundation for support as a fellow at the California Institute of Technology.

and OWEN 1959) in several respects and reflect the newly acquired information concerning the genetic relationships and distribution of the serotypes.

The inbred "families" of variety 1 have been established on the basis of the *mt* allele which they carry (NANNEY, CAUGHEY and TEFANKJIAN 1955; NANNEY 1959). Those with $mt^A$ are called "Family A"; those with $mt^B$ are called Family B, etc. With the identification of H serotypes, it became apparent that some of the sublines of these families, derived after intercrossing, were distinguishable from the primary stem lines. Moreover, additional sublines have been, and are being, developed deliberately to combine serotype and mating type characteristics. Therefore, when two sublines bearing the same *mt* alleles are serologically distinguishable, they are given different designations. For example, inbred series A and A1 have the same *mt* alleles but differ in serotypes. Families D and D1 are similarly distinguished.

The breeding procedures and culture methods are identical with those routinely used in Tetrahymena genetic studies (NANNEY and CAUGHEY 1955; NANNEY, CAUGHEY and TEFANKJIAN 1955). The procedures for preparing antisera and for identifying the serotypes are presented elsewhere (LOEFER and OWEN, in press).

## RESULTS

*The analysis of the Hc-Hd strain differences:* Serotype Hc was established in an inbred line by crossing the original strain manifesting the trait to an inbred Family D strain. This was followed by selective inbreeding from the progeny manifesting the Hc serotype. In the course of inbreeding, the mating type locus, $mt^B$, originally associated with the Hc serotype was replaced by the *mt* locus of Family D, $mt^D$. The new inbred series is called, therefore, Family D1. At various times in the breeding studies crosses were made back to the Family D strains and also to other strains showing the Hd serotype. Since the results of these various crosses are essentially identical, no effort is made to distinguish them in the following account.

Initially the exconjugants and caryonides of each pair were separated, allowed to give rise to small clones in depression cultures, and were then tested with both anti-Hc and anti-Hd sera. Since no marked differences could be detected among the progeny of single pairs, this practice was discontinued, and all the progeny of a pair were permitted to grow as a single culture. Within Family D1 all the progeny are Hc (72 pairs); within Family B all the progeny are Hd (98 pairs). All the offspring (76 pairs) of crosses between these two families are, when tested in the first depression cultures, immobilized or greatly retarded by both anti-Hc and anti-Hd serum. They are designated, therefore, as Hcd in phenotype.

The next step in the analysis was to cross the $F_1$ progeny and to ascertain the serotypes in the $F_2$. This could be accomplished only after the $F_1$ cultures were allowed to mature. The maturation was brought about by making single cell transfers from serial cultures on alternate days for 7–8 transfers. In most cases several different lines from each original pair were maintained to maturity. All the cultures were then tested for both mating type and serotype. These tests

yielded a surprising result. All the clones originally tested Hcd, but a large majority of the lines at maturity were either Hc or Hd. The remaining Hcd lines, showing responses to both antisera, were highly heterogeneous; in some, most of the cells gave slight reactions to anti-Hc and strong reactions to anti-Hd; others gave the reciprocal response or some response intermediate between the extremes. Even a single culture, tested with a single antiserum, showed some heterogeneity, in that the responses of individual cells were variable. The variability within a culture was, however, relatively much less obvious than that between cultures.

In spite of this complication, to which we will return later, the $F_2$ generation was produced and the progeny examined as before. Crosses were made between $F_1$ clones of various phenotypes to determine whether the phenotypic diversity was in any way significant in determining the outcome of the crosses. The results (Table 1) show that the inheritance of the H serotypes follows a simple Mendelian pattern; although the Hd phenotype is in slight excess, the P value for a 1:2:1 ratio is 0.1–0.2. The inbred Hc strains can be designated as $H^c/H^c$, the inbred Hd strains as $H^D/H^D$, and the $F_1$ clones, regardless of phenotype, as heterozygotes, $H^c/H^D$. It should be apparent, of course, that the genotype cannot be fully determined from the phenotype in mature cultures.

These results show that the phenotypes of the heterozygotes have no detectable influence on the phenotypes in the next generation. The genetic capacities, at least of the micronuclei, are not modified when a heterozygote undergoes the "differentiation" involved in changing its serotype expression. Two further kinds of crosses were undertaken to establish this point more firmly and to complete the evidence for a simple single gene control of antigenic potentialities. Crosses were made between heterozygotes in various phenotypic phases to homozygotes of various origin. The results (Table 2) are again consistent with the simple genetic model, and no evidence of phenotypic carry over is apparent.

*Evidence for additional alleles at the* H *locus:* Preliminary studies of crosses between strains manifesting other combinations of serotypes have been carried out. The most extensive of these studies involves the serotypes Ha and Hc. A representative of Family A showing serotype Ha was crossed with one from Family D1 showing serotype Hc. From a first cross, 41 pairs were isolated, and the first depression cultures were found to be affected by both anti-Ha and anti-Hc. Two lines were subsequently derived from each of the 41 pairs. In another

TABLE 1

*The results of crosses between* $H^c/H^D$ *heterozygotes manifesting various phenotypes*

| Parental serotypes | Serotypes of progeny | | | |
|---|---|---|---|---|
| | Hc | Hcd | Hd | Total |
| Hc × Hc | 35 | 70 | 51 | 156 |
| Hc × Hd | 29 | 65 | 34 | 126 |
| Hcd × Hd | 9 | 27 | 17 | 53 |
| Hd × Hd | 24 | 53 | 24 | 100 |
| Totals | 95 | 215 | 125 | 435 |

TABLE 2

*Results of crosses between homozygotes and* $H^C/H^D$ *heterozygotes showing various phenotypes*

| Parental genotypes | Parental phenotypes | Serotypes of progeny Hc | Hcd | Hd | Total |
|---|---|---|---|---|---|
| $H^C/H^C \times H^C/H^D$ | Hc × Hc | 32 | 33 | 0 | 65 |
| | Hc × Hd | 12 | 12 | 0 | 24 |
| Totals | | 44 | 45 | 0 | 89 |
| $H^C/H^D \times H^D/H^D$ | Hc × Hd | 0 | 16 | 15 | 31 |
| | Hcd × Hd | 0 | 16 | 13 | 29 |
| | Hd × Hd | 0 | 17 | 19 | 36 |
| Totals | | 0 | 49 | 47 | 96 |

experiment, both exconjugant clones from 26 pairs were examined and found to react to both antisera. As in previous crosses, the initially double response disappeared in many of the sublines during the growth to maturity. After nine transfers, and about 120 cell divisions, only 21 of the 133 lines followed were still Hac. The ratios of the two pure types were eccentric; 103 of the pure lines were Ha and only nine were Hc.

Various crosses were made among the $F_1$ clones, and the serotypes of the $F_2$ clones were determined as before. The $F_2$ pairs presented certain complications in classification not encountered in the Hc–Hd study. Three major classes of pairs were again discerned, those in which both initial exconjugant clones were Ha, those in which both clones were Hc, and those in which the progeny manifested some degree of reaction to both anti-Ha and to anti-Hc. However, these latter pairs were heterogeneous; in some the two responses were clear-cut for both exconjugant clones; in some the reactions to anti-Hc were weak in one or both clones, and, in a few, one clone tested Hac and the other Ha. Nevertheless, the ratios of the three classes of $F_2$ pairs (Table 3) correspond to a 1:2:1 phenotypic ratio, again indicating a single gene difference between strains pure for the Ha and strains pure for the Hc serotypes. As in the previous study, no influence of the phenotype of the parent on the phenotypes of the progeny is apparent. One small-scale backcross of a heterozygote to the Hc parent yielded 14 type Hc progeny

TABLE 3

*The results of crosses between* $H^A/H^C$ *heterozygotes manifesting various phenotypes*

| Parental serotypes | Serotypes of progeny Ha | Hac | Hd | Total |
|---|---|---|---|---|
| Ha × Ha | 18 | 31 | 17 | 66 |
| Ha × Hc | 17 | 30 | 12 | 59 |
| Hc × Hc | 5 | 16 | 7 | 28 |
| Totals | 40 | 77 | 36 | 153 |

and 13 type Hac. It appears reasonable to conclude, therefore, that the Ha serotype is determined by an allele at the *H* locus, to be designated as $H^A$.

All the $F_2$ and backcross cultures in this study were maintained by single-cell isolations through about 120 cell divisions and again tested for serotypes. While the initially pure Ha and Hc cultures remained unchanged, most of the cultures having the Hac phenotype became pure Ha or pure Hc. The ratio of the two types was eccentric; as in the $F_1$, the Ha type was 5–6 times as frequent as the Hc type.

Mention must also be made of certain aberrant $F_2$ clones not included in the above tabulations. One clone failed to show reactions to either anti-Ha or anti-Hc in repeated tests. Three showed typical Ha reactions except for a slight but significant retardation in anti-Hd. Four showed good reactions to anti-Hc, but also showed reactions to anti-Hb and anti-Hd. Several explanations for these aberrant clones are possible, but further tests are required to establish or to disprove them.

Finally, a few additional crosses have been made to further associate the various H serotypes. Twenty pairs from a cross between pure Ha and pure Hd clones were examined and found in the first depression to be Had. A total of 120 sublines were then followed to maturity and retested. All except eight had become Ha; the remaining sublines consisted of five type Hd and three type Had. In the same manner eight $F_1$ pairs from Hc × He were studied. In the first depressions, all showed strong reactions to anti-He but weak and variable reactions to anti-Hc. From the expanded lines (120), all eventually became pure He except four which became Hc.

Although incomplete, the available information is consistent with the hypothesis that the H serotypes are associated with multiple alleles at a single locus, the *H* locus. The $H^A$, $H^C$ and $H^D$ alleles are the most firmly established. An $H^E$ allele is suggested, and a distinctive allele, $H^B$, may exist in the sterile wild strain, WH-52. All heterozygous combinations appear to be associated with a peculiar variability of phenotypic expression; some sublines "differentiate" to express the activity of one allele, and other sublines differentiate to express the other. Differences in the relative probabilities of expressing (or excluding) particular specificities are apparent. These may depend upon the combination of alleles, background genetic effects, or perhaps environmental variables. In the situations thus far tested, however, the phenotypic differentiation in one generation appears not to be significant in determining the differentiation in the next.

*The mechanism of vegetative assortment of H serotypes:* The crucial problem exposed in the previous sections is the vegetative change of phenotypes associated with heterozygosis at the *H* locus. An evaluation of alternative interpretations of the phenomenon requires a fuller description than has thus far been provided. All the studies to be reported refer specifically to the Hc and Hd serotypes, but preliminary observations on the other dual serotypes provide qualitative confirmation.

To provide quantitative data on the serotypic changes, 16 different caryonidal cultures manifesting the dual serotype Hcd were expanded to provide a total of 240 sublines. After two-days growth each of these sublines was reinitiated from a single cell, and the process was continued serially. After each reisolation the

"leftover" culture was tested by mixing one drop of the culture with anti-Hc serum and one drop with anti-Hd serum in concentrations sufficient to immobilize pure cultures in 30–60 minutes. The tests were read at about 45 minutes.

Considerable diversity of response was observed, and an arbitrary classification was devised. The range of responses constituted a continuous series from complete immobilization in anti-Hc and no reaction to anti-Hd (c responses) to the opposite extreme (d responses). The intermediate responses, immobilization or retardation of most of the tested population in both antisera, could be classified into three major categories. A *cd* response indicates a stronger response to anti-Hc than to anti-Hd; a cd response indicates essentially equal effects of the two sera; *cd* responses are predominant responses to anti-Hd. Certain difficulties are inherent in this classification. The *cd* responses are difficult to distinguish from pure c responses at one extreme and from cd responses at the other. Similarly, *cd* responses grade into both d and cd responses. The breadth or narrowness of the cd category depends on how fine a distinction one wishes to make. Nevertheless, the classification scheme serves a useful descriptive purpose. The classified data from the entire series are summarized in Table 4. The departures from a total of 240 clones classified reflect the clones which died or were so degenerate that classification was difficult. When clones died, they were replaced by isolations from sister cultures which were vigorous.

The general features of the time course of serotype change are clear. Cells isolated from cultures 18-fissions-old usually produce cultures giving intermediate responses. Upon further isolation, however, smaller and smaller fractions give intermediate responses. At first the pure cultures are mainly type Hd, but eventually pure cultures of type Hc are produced.

The individual subseries, derived from different caryonides, do not behave uniformly. A few produced type Hd exclusively and quickly; most produced a larger fraction of Hd than of Hc, but a few gave more Hc than Hd.

The array of serotypes at any one time does not provide all the information needed for an analysis. An examination of individual pedigrees is required to determine the stability of the phenotypes. All subcultures of a particular phenotype, regardless of their positions in the pedigrees, were grouped into a single

TABLE 4

*The distribution of serotype phenotypes in subclones derived from single cells at various times since conjugation*

| Phenotypes | Fissions since conjugation 18 | 31 | 44 | 57 | 70 | 83 | 96 | 109 | 122 |
|---|---|---|---|---|---|---|---|---|---|
| c | 0 | 15 | 17 | 20 | 29 | 42 | 41 | 40 | 41 |
| *cd* | 1 | 9 | 25 | 42 | 35 | 18 | 14 | 18 | 18 |
| cd | 6 | 58 | 35 | 20 | 17 | 13 | 13 | 12 | 11 |
| *cd* | 4 | 33 | 40 | 33 | 19 | 17 | 24 | 20 | 9 |
| d | 5 | 123 | 120 | 126 | 139 | 144 | 147 | 149 | 157 |
| Totals | 16 | 238 | 227 | 231 | 239 | 234 | 239 | 239 | 236 |

class, and the phenotypes of their next serial derivatives were examined. The intermediate categories (*c*d; cd; and c*d*) each produced progeny with scattered phenotypes, centering about the phenotype of the original culture, but including less than 50 percent in that class. The intermediate phenotypes show, therefore, only moderate heritability. In contrast, the cultures at the extremes of the range, the pure c and pure d cultures, produce largely cultures of the same type, and only occasionally produce derived cultures of intermediate rank. Two *a priori* interpretations of these "reversions" are possible; they could represent imperfect classification of the cultures so that occasional *c*d cultures are classified as c, and some c*d* cultures are classified as d. In this case their production of intermediate phenotypes in derived cultures would not represent true instability but only the imperfections of the techniques. The occasional misclassification of clones can scarcely be doubted. The more crucial question is whether some "reversions" are real, i.e., whether clones which have for a period of time produced no antigen of a particular kind can reinitiate its production. In this latter case one might expect that any pure culture regardless of the time since it became pure would have a constant probability of producing intermediate derivatives. If, the reversions are artifacts, the more times a culture had been tested, the smaller should be the possibility that it is erroneously classified and the smaller should be the probability that it will yield intermediate derivatives. To test this point, all pure cultures were classified according to the number of times in sequence they had tested pure. This gives an array of "pure" cultures ranging from those which had been so classified for the first time through those which had been classified pure for as many as ten successive transfers. The behavior of the next serial isolation in each case was noted (Table 5). Clones classified as pure for the first time revert in over 20 percent of the cases; those classified pure for the second time yield intermediate progeny in less than ten percent. Less than one percent revert after five pure classifications, and no reversions were found after six or more pure classifications. These results are consistent with the hypothesis that the reversions are artifacts of the techniques, reflecting the limitations of the immobilization tests. The possibility of true reversions still exists, but thus far no evidence for them has been obtained. If they occur at all, they must occur at a very low rate, at least after a period of "pure" growth. These observations also provide an opera-

TABLE 5

*The phenotypes of the next isolation cultures in lineages which had tested "pure" for different numbers of transfers*

| No. of previous pure cultures | Phenotypes of derived cultures | | |
|---|---|---|---|
| | Pure | Intermediate | Percent intermediate |
| 1 | 205 | 56 | 21.5 |
| 2 | 225 | 23 | 8.9 |
| 3 | 226 | 9 | 3.8 |
| 4 | 183 | 6 | 3.2 |
| 5 | 187 | 1 | 0.5 |
| 6 or more | 505 | 0 | 0.0 |

tional definition for fixation: clones which have tested pure for six successive serial cultures are certainly pure; those which have tested pure for even three or four transfers have a very high probability of being pure.

This analysis permits a study of the kinetics of the fixation process, even though certain problems remain. A clone which has been classified as pure in six or more serial cultures is certainly pure in the terminal culture, but the precise time in the sequence that it became pure cannot be determined with precision. In some cases it must have been pure the first time it so tested; in other cases it must not have become pure until after one or more further transfers. If all fixations are assumed to have occurred at the initiation of the first pure culture, fixations are actually scored in some instances prior to their occurrence, and the absolute time axis in a fixation curve is slightly distorted. If, however, one is primarily interested in absolute rates of fixation, this distortion is of little consequence so long as the fraction stabilizing is small. In any case, adjustments can be made when they seem to be indicated.

For the following tabulations $R_t$ (Rate of fixation per transfer) is defined as the fraction of cells isolated at any time from cultures of intermediate phenotype which yield persistently pure cultures in further transfers (in most cases six or more transfers, but occasionally only three–five when a culture was lost before completing the sequence).

$R_t$ has two components, $R_{tc}$ and $R_{td}$, representing the rates of fixation to Hc and Hd, respectively. $R_t$ is found (Table 6) to remain approximately constant throughout the interval investigated at a value of $0.17 \pm 0.02$. The components of $R_t$ vary, however, with $R_{td}$ starting high and gradually becoming smaller and $R_{tc}$ beginning at a very low figure and rising gradually until it is equal to $R_{td}$. No tests of partial rates beyond the convergence point have been made; conceivably, the rates cross, but this is considered unlikely. The rate per transfer, $R_t$, can be converted into a rate per fission, $R_f$, by the simple expedient of dividing the rate per transfer by the number of cell divisions occurring before transfer. Under the conditions employed in these experiments (transfers on alternate days), the number of cell divisions between transfers is about 13, and $R_f$ has a value of approximately 0.013. This is a significant figure to which we will return subsequently.

A very similar pattern of vegetative assortment of phenotypes has already been studied in these strains (ALLEN and NANNEY 1958) during examination of the mating type system. Since the two systems appear so similar, it is important to

TABLE 6

*The relationships between rates of fixation of types and fissions since conjugation*

| Rates | Fissions since conjugation 18 | 30–56 | 69–94 | 107–133 | Total |
|---|---|---|---|---|---|
| $R_{tc}$ | .00 | .032 | .040 | .105 | 44/935 |
| $R_{td}$ | .19 | .137 | .107 | .088 | 115/935 |
| $R_t$ | .19 | .169 | .147 | .193 | 159/935 |

determine whether they are indeed quantitatively alike. The rate of mating type fixation per fission ($R_f$) had been found to be about 0.0113, slightly lower than the $R_f$ determined for the serotype system. These figures are not, however, directly comparable since the conditions of the experiments were slightly different. In the mating type studies the transfers were made daily to prevent the selfing which occurs in lineages unstable for mating type and to avoid the slight starvation encountered with alternate day transfers. Severe starvation had been found to increase the rate of fixation in some clones. To provide more nearly comparable data an additional experiment was designed, identical to the previous one in general procedures, but involving daily transfers of single cells. In this experiment 39 fixations were scored for 433 opportunities, yielding an $R_t$ of $0.090 \pm 0.014$. Since the cells undergo about eight fissions per day under these conditions the $R_t$ is about $0.0112 \pm 0.0018$, indistinguishable from the $R_f$ of $0.0113 \pm 0.0004$ (calculated from Table 1, ALLEN and NANNEY 1958) in the mating type system.

This experiment was also designed to provide information on the behavior of sister caryonides from a single pair. Each caryonide in the previous experiment was taken from a different pair, and one could not determine whether the differences in frequencies of the pure types at the end of experiment were due to differences between pairs or could arise within a single pair. The latter experiment included all four caryonides from three different pairs. The distributions of serotypes in the terminal cultures from different caryonides are given in Table 7. Although all caryonides showed evidence for some Hc and some Hd antigen, the relative amounts in the different caryonides were strikingly different. Caryonide 1a, for example, was predominantly type Hc in the terminal cultures while caryonide 1b, a sister caryonide from the same pair, produced more pure Hd lines than pure Hc lines. Similar observations hold for caryonides 3a and 3b. These differences cannot reflect differences in initial genetic constitutions and must

TABLE 7

*The distribution of phenotypes among lineages derived from related caryonides*

| Caryonide | Phenotypes of sublines | | | | | |
|---|---|---|---|---|---|---|
| | c | *cd* | cd | *cd* | d | Total |
| 1a | 20 | 7 | 0 | 0 | 1 | 28 |
| 1b | 4 | 10 | 2 | 1 | 13 | 30 |
| 1c | 9 | 5 | 1 | 1 | 7 | 23 |
| 1d | 7 | 18 | 4 | 1 | 0 | 30 |
| 2a | 18 | 6 | 2 | 0 | 1 | 27 |
| 2b | 12 | 5 | 2 | 0 | 2 | 21 |
| 2c | 18 | 4 | 1 | 2 | 2 | 27 |
| 2d | 7 | 12 | 7 | 0 | 3 | 29 |
| 3a | 3 | 9 | 0 | 1 | 15 | 28 |
| 3b | 22 | 6 | 1 | 1 | 0 | 30 |
| 3c | 24 | 1 | 2 | 1 | 2 | 30 |
| 3d | 12 | 6 | 0 | 1 | 7 | 26 |

reflect differences which arise subsequent to the establishment of the fertilization nucleus. Such a conclusion does not eliminate the possibility that initial genetic differences may influence the probabilities for different kinds of "differentiation." Indeed, it should be noted that most of the first caryonides studied produced predominately Hd lines in the terminal cultures, while the second cross produced predominantly Hc pure lines. The factors, genetic and environmental, significant for these differences will have to be explored systematically.

## DISCUSSION

*The mating type and serotype systems in variety 1:* The interpretation of the results relies heavily upon earlier studies of intraclonal variation in ciliates, particularly on serotype determination in Paramecium (BEALE 1957; SONNEBORN 1957) and mating type determination in Tetrahymena (NANNEY 1958). It is necessary, therefore, to point out the essential features of these earlier analyses. The most direct parallel is between the mating type and the H serotype systems in variety 1 of *T. pyriformis*.

All the available inbred strains of variety 1, although essentially homozygous, continue in each generation to produce an array of mating types. The arrays in the different "families" are, however, distinctive, either in regard to the types which are expressed or the frequencies in which the types are produced (NANNEY, CAUGHEY and TEFANKJIAN 1955; NANNEY 1959). Genetic analysis of strain differences locates the major determinants of mating type specificities at a single chromosomal locus, designated as the *mt* locus.

The manner in which diverse mating types are distributed among the progeny of a single pair are instructive in defining the physical basis of the mating type differences. As SONNEBORN demonstrated long ago in Paramecium (SONNEBORN 1937, 1947, 1957), mating type diversities assort in Tetrahymena primarily at the time new macronuclei are assorted following conjugation. The distribution of mating types among the progeny of a pair demonstrates that the mating type differences among cells of identical genotype are localized within the macronucleus, and mating type determination must be viewed as a process of nuclear differentiation (NANNEY 1956, 1958).

Further insight into the nature and localization of the macronuclear differentiation came from an analysis of clones (caryonides) which were unstable in their mating types and produced pure derivatives during cellular proliferation (NANNEY and CAUGHEY 1955; ALLEN and NANNEY 1958). Most unstable lineages are capable of producing two pure mating types, but any combination of two mating types may be produced in different unstable clones. Regardless of the types produced, the kinetics of the fixation process appear identical. Specifically, after an initial period of variability, all clones begin to produce pure types at a rate calculated to be 0.0113/cell division. Furthermore, the components of this rate (the rates to the two different mating types being produced) are often initially very unequal but gradually approach equality in a time course experiment.

To explain these observations, a particulate segregation model was developed (SCHENSTED 1958), and new experiments were designed to test its applicability. Thus far all the data are consistent with the following hypotheses: (1) the macronucleus consists of a population of diploid subnuclei; (2) the subnuclei are capable of differentiating to yield a heterogeneous macronucleus; (3) once differentiated the subnuclei breed true and are assorted approximately at random to the daughter nuclei produced at fission. One significant property of the model is the relationship between the number of assorting subnuclei (N) and the equilibrated fixation rate ($R_f$), shown to be $R_f = \frac{1}{2N-1}$. The number of subnuclei in a newly divided cell is calculated to be about 45.

The studies on the H serotype system show remarkable similarities to those on the mating type system. Again the specificities for a particular kind of trait are associated with a particular genetic locus, in this case the *H* locus. Again some mechanism of mutual exclusion prevents the persistent expression of all specificities simultaneously. The differences in the behavior of sister caryonides suggests, as for the mating type system, that the critical differentiations are macronuclear, but further studies are required to establish this point with certainty. In both cases, pure lines of cells are produced during cellular proliferation, and these maintain their phenotypes during further cell division. Most significantly, the rates of production of pure lineages in the two systems are apparently the same, about 0.0113/cell division, and the same kind of covariation of partial rates of fixation is observed. Finally, in neither case do the differentiations occurring in one sexual generation influence the differentiations in the next generation.

In the face of these parallels, one can scarcely doubt that a similar mechanism underlies the two kinds of differentiation. If the vegetative assortment of mating types is to be explained by the assortment of differentiated diploid subnuclei, then the vegetative assortment of serotypes must also be explained in this way. The simplest interpretation of the serotype system involves the following postulates: (1) that the macronucleus consists of a population of diploid subnuclei, (2) that the subnuclei become differentiated by the suppression of one of the pair of alleles in each subnucleus; (3) that once established, the differentiations are perpetuated indefinitely in further subnuclear replications, and (4) that pure lines are produced by the random assortment of differentiated subnuclei. The interallelic suppression may be viewed as kind of "persistent indeterminate dominance."

This interpretation is predicated on a particular view concerning the organization of the ciliate macronucleus, specifically the idea that the macronucleus consists of many discrete diploid subnuclei. Alternative views are that the subnuclei are, or can become, haploid, or even that subnuclei are nonexistent and the macronucleus is simply a disorganized bag of individual chromosomes. If diploid genomes are disrupted and an assortment of chromosomes or genomes occurs during cellular division, one would expect that any heterozygote could be resolved into its components by continued cellular division. The behavior of the serotypes in Tetrahymena would then simply reflect a process of chromosomal segregation common to all macronuclear genes. This explanation cannot be ruled out directly

for the serotypes, but it is inapplicable to the mating type system. Somatic assortment occurs for mating types in nearly all newly constituted clones, even after ten generations of inbreeding by crosses of sister caryonides—at a time when heterozygosis is extremely improbable. Moreover, the production of three or four different mating types from some caryonides could not be explained on the basis of a single pair of segregating alleles.

Unfortunately, a direct test for segregation of other genes in the macronucleus of Tetrahymena is not yet possible, and most of the arguments for intact diploid genomes are indirect and fail to be compelling. It is necessary, therefore, to rely on the studies on the one ciliate which has been examined intensively on this point. Exhaustive attempts to demonstrate macronuclear segregation in *Paramecium aurelia* (SONNEBORN, SCHNELLER and CRAIG 1956), utilizing a variety of different genes, have failed to reveal a single case of genetic assortment. SONNEBORN did observe certain serotypic variants comparable in some ways to those reported here, but in no case was the ability to produce a particular antigen lost completely or irrevocably. Unless the macronucleus in Paramecium is organized on a different plan from that in Tetrahymena, we must conclude that diploid genomes are usually maintained intact, and that the phenotypic variants observed in Tetrahymena reflect nuclear differentiation rather than genetic recombination.

One further word should be said concerning the relationship between the serotype and mating type systems in variety 1. The many similarities might suggest that they are indeed the same systems assayed in different ways. This contention can be disproved by a detailed comparison, but more compelling information is being developed. Preliminary studies (NANNEY, unpublished) indicate that the *H* and the *mt* loci are unlinked and that differentiations in one system are independent of those in the other system. The parallels are not, therefore, based on a single common mechanism but are based on the operations of similar but separate mechanisms.

*The serotype systems in Tetrahymena and Paramecium:* The serotype system in Tetrahymena shows many similarities to the serotype system in Paramecium (BEALE 1954, 1957; SONNEBORN 1957) but also some significant differences. In Paramecium a single clone has a wide variety of antigenic potentialities but normally expresses only one at a time. The antigens which are expressed can often be called forth by particular modifications in the environment, but intraclonal variants may persist for long periods of time in the same environment. Genetic analyses of antigenic potentialities show that a large number of independent genes are involved. Serotype differentiation may be viewed, therefore, as a special kind of "conditional epistasis"; the expression of a gene at one locus ordinarily prevents the expression of genes at other loci. On the other hand, in Paramecium the expression of a gene does not usually prevent the expression of an allelic gene; indeed, with a few conspicuous exceptions (SONNEBORN, SCHNELLER and CRAIG 1956; FINGER 1957), the two alleles at a given locus are expressed simultaneously, no matter how diverse their products may appear serologically. Finally, crosses between strains of identical genotype manifesting

different serotypes show that the cytoplasm plays an important role in maintaining serotypic "states."

In Tetrahymena (LOEFER, OWEN and CHRISTENSEN 1958; MARGOLIN, LOEFER and OWEN 1959; INOKI and MATSUSHIRO 1958), many of the same observations are available. Again a single clone can differentiate into a variety of serotypes, but a particular cell normally expresses only one antigen at a time. Differentiated subclones can be maintained for a period of time in the same environment. Thus far the genetic basis for only the "H" serotypes has been examined; whether the genes for the other serotypes are linked or are widely distributed is not yet known. Within the H system at least, the interallelic relations appear distinct from those usually encountered in Paramecium; in Tetrahymena the exclusion of diverse serotypes seems to hold not only for nonallelic genes but even for allelic genes. Whether allelic exclusion operates at other loci has not yet been determined, but it appears to operate in all allelic combinations for the *H* locus.

The available information, though meager, suggests that the mechanisms of interlocus exclusion and interallelic exclusion are distinct. The interallelic exclusion appears to be a strictly nuclear phenomenon; no evidence for a cytoplasmic carry-over from one generation to the next has been found. In contrast, the one breeding study involving presumably interlocus exclusion (INOKI and MATSUSHIRO 1958) suggests that, as in Paramecium, the cytoplasm can determine the phenotype of a new cell following conjugation. This study was, however, a small scale study and certain necessary precautions against "nonconjugation" were not reported. Further work will be necessary before this point can be considered established. Another significant difference between the interallelic and interlocus differentiations is their relative reversibility. No cases of reversion of interallelic differentiations have been encountered, if one accepts the interpretation that early "reversions" actually represent cases of misclassification. Interlocus differentiations are, on the other hand, freely reversible. Preliminary observations (NANNEY, unpublished) suggest that the interallelic differentiations persist even during a period when the locus is not being expressed, i.e., when a cell in the "H" state is transformed to the "L" state and back again. Quite possibly the two systems of serotype differentiation find parallels in the two major classes of mating type differentiation in the ciliates—the group A system being localized strictly within the nucleus, and the group B system having a major cytoplasmic component in its mechanisms of perpetuation (SONNEBORN 1954, 1957; NANNEY 1954, 1958).

## SUMMARY

Strains of variety 1 of *T. pyriformis* manifest different immobilizing antigens when grown at 25°C in an Aerobacter-Cerophyl medium. Breeding analyses show that the ability to produce these particular antigens, the H antigens, is associated with a single genetic locus, the *H* locus. Four distinctive alleles, $H^A$, $H^C$, $H^D$, and $H^E$, appear to be established in different inbred strains.

Although the transmission of antigenic potentialities in sexual processes is

simple and uncomplicated, the expression of the *H* alleles poses problems. Heterozygous clones initially express both antigenic specificities, but in the course of cellular proliferation they produce some sublines expressing only one of the antigens and others expressing only the other. The phenotypically "pure" lineages maintain their characteristics in further growth. The kinetics of antigenic fixation are identical with the kinetics of mating type fixation in clones of "unstable" mating types, and a similar explanation must be applied to both. The preferred explanation for serotype "differentiation" is that one allele in each subnucleus of the compound macronucleus is suppressed, that the suppression becomes an hereditary character of the subnucleus and that the fixation process is the result of an assortment of differentiated subnuclei at macronuclear division. The phenomenon is considered as a kind of "persistent indeterminate dominance." The differentiations in one sexual generation appear not to influence the differentiations in a subsequent generation.

The system of mutual exclusion among the H serotypes is not to be confused with the system of mutual exclusion of presumably nonallelic antigen determining genes, which has very different properties. Specifically, the interlocus differentiations appear much more readily reversed and probably involve a cytoplasmic component in a feedback system.

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# THE RELATIONSHIP BETWEEN THE MATING TYPE AND THE H SEROTYPE SYSTEMS IN TETRAHYMENA

D. L. NANNEY[1]

*Zoology Department, University of Illinois, Urbana, Illinois*

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TWO systems of cellular differentiation in variety 1 of *Tetrahymena pyriformis* are remarkably similar (ALLEN and NANNEY 1958; NANNEY and DUBERT 1960). In both cases cells of the same clone, and presumably of the same genotype, may come to manifest highly distinctive cellular phenotypes, even when grown in identical environments. In both cases sublines of a clone gradually diverge and eventually become clearly and persistently distinguishable. The detailed kinetics of the "fixation" of pure types in the two systems, so far as they have been studied, are alike; after an initial period of equilibration the rate of fixation is about 0.011 per cell division. In one case (mating types) compelling evidence is available for a macronuclear basis for the variation; suggestive evidence for a macronuclear basis of serotype differences has been reported. Finally, single chromosomal loci appear to set the limits of variation in the differentiations, specifically, the *mt* locus for the mating types and the *H* locus for the serotypes.

The similar behavior of the two systems requires a similar explanation. The interpretation proposed and supported by previous studies is that diploid subnuclei become differentiated during the development of the macronucleus, that these differentiations become hereditary characteristics of the subnuclei, and that the fixation of pure sublines results from the random assortment of differentiated subnuclei at cell division, a process of "intranuclear genetic drift." The segregation model (SCHENSTED 1958) permits an estimate of the number of assorting units, about 45 in newly divided cells.

In spite of the similarities in the two systems, a detailed comparison demonstrates some significant differences between them. For example, the somatic variation in H serotypes appears to be restricted to heterozygotes, but in the mating types occurs in both homozygotes and heterozygotes. Moreover, only two stable types, corresponding to the specificities of the two alleles present, have been extracted from a single clone showing serotype variation; but in rare cases as many as four distinctive mating types have been extracted from a single clone (caryonide). These observations suggest that the details of subnuclear differentiation are different in the two cases, and that the similarities noted merely reflect a common mode of assortment of differentiated subnuclei. To further elucidate the relationship between these two systems, experiments were designed to answer two questions: (1) What is the genetic relationship between the *H* and the *mt* loci? and (2) Are serotype and mating type differentiations correlated in any way?

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## MATERIALS AND METHODS

Two sets of strains were employed in this study, representatives of an inbred series designated as Family D1 ($mt^D/mt^D$; $H^C/H^C$) and representatives of an inbred series designated as Family B ($mt^B/mt^B$; $H^D/H^D$) (NANNEY and DUBERT 1960). Strains homozygous for the $H^C$ allele, when grown at 25°C in an Aerobacter-Cerophyl medium, are uniformly immobilized by homologous antiserum; they are said to manifest the Hc phenotype. Similarly, strains homozygous for the $H^D$ allele are immobilized under the same conditions by their homologous antiserum and are designated as Hd in phenotype (LOEFER and OWEN, in press). The two antisera show essentially no cross-reactions at the concentrations employed. The $F_1$ hybrids between the strains are initially immobilized by either antiserum, and their phenotype is indicated by the symbol Hcd. Following successive single cell transfers, however, the heterozygotes eventually produce many phenotypically pure sublines of type Hc and Hd. The initial phenotypes of the $F_2$ are Hc, Hcd and Hd, in approximately a 1:2:1 ratio.

The mating type genotypes are determined with much greater difficulty than the serotype genotypes. A particular allele potentiates the production of five or six different mating types in various frequencies, and only a fraction of the possible mating types usually appear among the progeny of a single pair. Moreover, the mating types of the progeny cannot be ascertained until after a period of sexual immaturity lasting 60 or more cell divisions. A total of four different alleles of the *mt* gene have been established in inbred lines (NANNEY 1959), but only three have thus far been involved in breeding analyses: $mt^A$, $mt^B$ and $mt^C$. A secondary purpose of these experiments was to obtain segregation data for the $mt^D$ allele; unfortunately, this allele is the most difficult of the four to identify.

The gene $mt^B$ potentiates the production of the six mating types—II, III, IV, V, VI and VII. The $mt^D$ allele, on the other hand, makes possible the production of types I, II, III, V and VI. The heterozygote may produce any of the seven types. The genotype of an $F_2$ segregant is ascertained by its manifestation—either directly in the $F_2$ or in progeny tests—of the critical types I, IV and VII. If type IV or VII is produced, at least one $mt^B$ allele is present. Similarly, if type I appears, $mt^D$ must have been present. If both I and IV or VII appear, the pair is classified as a heterozygote, $mt^B/mt^D$.

Establishing the *absence* of an allele is a more complicated procedure and requires progeny tests. Progeny tests of two kinds have been used. The most efficient tests are those by inbreeding from a single $F_2$ pair to produce an $F_3$. This is possible only when two or more mating types are present in the $F_2$ and when the cross is fertile. If inbreeding cannot be carried out, backcrosses to the parental strains can often be employed, but this requires at least twice as many progeny to yield the same result. Backcrosses to the $mt^B/mt^B$ parent yield information only regarding the ability of the tested clone to produce type I, i.e., they determine the presence or absence of the $mt^D$ allele. Backcrosses to the $mt^D/mt^D$ parent yield information only about the presence of the $mt^B$ allele.

The reliability of progeny tests depends on two factors—the size of the sample

and the probability of expression of the critical mating types. The absence of $mt^B$ can be established with relative ease because either type IV or VII indicates its presence; and type IV is a common type, being produced in nearly 50 percent of caryonides of homozygotes and about half this frequently in most heterozygotes. Hence, if neither IV nor VII appears in a progeny test of 20–30 caryonides, one may be reasonably certain that $mt^B$ is absent from the tested zygote.

In contrast, the inbred $mt^D/mt^D$ strains used in these crosses produce only about 14 percent of type I, and in heterozygotes with $mt^B$ even fewer might be produced. Establishing the absence of the $mt^D$ allele would require large progeny tests and was not considered necessary in these studies, particularly since the alternative allele could be scored readily. Although no systematic effort was made to determine the distribution of the $mt^D$ allele among the $F_2$ pairs, direct evidence for its presence was sometimes obtained incidentally while ascertaining the $mt^B$ distribution. A more efficient study would have employed $mt^C$ (producing 60 percent type I in some homozygotes) or $mt^A$ (producing about 25 percent type I in some homozygotes).

## RESULTS AND ANALYSIS

*Genotype distributions:* A cross was made between strains of genotype $H^C/H^C$; $mt^D/mt^D$ and $H^D/H^D$; $mt^B/mt^B$. Three $F_1$ pairs of the genotype $H^C/H^D$; $mt^B/mt^D$ were selected to produce the $F_2$. No attempt was made to isolate the exconjugants and caryonides of the $F_2$ pairs, and all the progeny of a single pair were allowed to grow in a single culture. After routine tests for "nonconjugation," the initial cultures were tested with both anti-Hc and anti-Hd serum and classified on the basis of their responses. Of the 122 pairs examined, 29 were Hc, 65 were Hcd and 28 were Hd. Since the phenotype of *initial* cultures is directly related to their genotype, the "*H*" genotypes of the $F_2$ pairs were immediately assigned, i.e., $H^C/H^C$, $H^C/H^D$, and $H^D/H^D$. The 1:2:1 ratio ($P = 0.7$–$0.8$) confirms previous studies on the segregation of these alleles.

The remaining, and more difficult, problem was to determine the *mt* genotypes. The first approach to this objective consisted of isolating nine individual cells from the first cultures produced by each of the 122 pairs, to continue these secondary lines through eight serial transfers, and to test the terminal mature cultures for mating type. If all the four independently differentiated caryonides are represented in the sample from a pair, most pairs should be assignable to a "partial genotype." Unfortunately, considerable caryonidal death or differential growth must have occurred; many of the pairs were represented by nine sublines of a single mating type. Nevertheless, partial genotypes could be assigned to many of the $F_2$ pairs on the basis of the $F_2$ phenotypes. Those pairs which produced IV or VII, but no I, were of genotype $mt^B/?$; those which produced I, but no IV or VII, were of genotype $mt^D/?$; those producing both I and IV or VII were completely defined as $mt^B/mt^D$. Unfortunately, 38 of the 122 pairs produced neither I nor IV or VII. A preliminary test of correlation showed no relationship between the *H* genotypes and these partial *mt* genotypes.

To refine further the genotypic determinations, progeny tests (either $F_3$ or backcrosses to Family D1) were carried out on 16 of the 17 $mt^D/?$ pairs and on the 38 pairs for which no information was provided in the $F_2$ tests. One $mt^D/?$ pair could not be tested. The progeny tests consisted of the progeny of 30 pairs, usually with only one subline representing a pair. If neither type IV nor VII appeared in the $F_2$ or the progeny tests, the pair was classified as $mt^D/mt^D$. If both I and IV or VII appeared under proper circumstances, the pair was classified as $mt^B/mt^D$. No effort was made to resolve the $mt^B/?$ pairs. This procedure established three categories of $F_2$ pairs: $mt^D/mt^D$, $mt^B/mt^D$, and $mt^B/?$. The frequencies found were 22:28:72, respectively. Since the $mt^D$ segregation was not completely analyzed, the only test of significance for this distribution is that on the partition of $F_2$ pairs with at least one $mt^B$ allele against those with no $mt^B$ allele. The observed distribution of 100:22 is only a fair fit to a 3:1 ratio ($P = 0.05$–$0.10$).

Even after this refinement of the *mt* genotypes, no preferential association of *mt* and *H* genotypes could be detected (Table 1). The three *H* genotypes were distributed in an approximate 1:2:1 ratio in each of the *mt* genotypes ($P = 0.9$–$0.95$). Even if the distributions are lumped by pooling the classes expected to be large on a linkage hypothesis ($mt^D/mt^D$; $H^C/H^C$:$mt^B/mt^D$; $H^C/H^D$; and $mt^B/?$; $H^D/H^D$) against those with expectedly low values (the remaining genotypes), the P value is still 0.5–0.7. Thus, no evidence for linkage is provided, and any reasonably close linkage is rendered improbable. The *mt* and *H* loci are transmitted essentially independently.

*Phenotype distributions:* Even though the *H* and *mt* genotypes show no preferential association in the $F_2$ of a dihybrid cross, the possibility remains that a phenotypic association exists between the H serotype and the mating type systems. For example, one might find in lineages giving "vegetative assortment" of both mating types and serotypes that certain mating types would be associated with a particular serotype more often than expected by chance. This in turn would suggest that the conditions responsible for particular serotype and particular mating type determinations were related. The experiment reported in the previous section presented an opportunity to test this possibility.

TABLE 1

*The distribution of* H *genotypes among partial* mt *genotypes*

| Partial *mt* genotypes | Serotype genotypes | Found | Expected |
|---|---|---|---|
| $mt^D/mt^D$ | $H^C/H^C$ | 7 | 5.5 |
| | $H^C/H^D$ | 11 | 11 |
| | $H^D/H^D$ | 4 | 5.5 |
| $mt^B/mt^D$ | $H^C/H^C$ | 7 | 7 |
| | $H^C/H^D$ | 15 | 14 |
| | $H^D/H^D$ | 6 | 7 |
| $mt^B/?$ | $H^C/H^C$ | 15 | 18 |
| | $H^C/H^D$ | 39 | 36 |
| | $H^D/H^D$ | 18 | 18 |

The 65 $F_2$ pairs of genotype $H^C/H^D$ assorted serotypes during the growth to maturity; they had also undergone mating type differentiation, and the survivors of the original nine isolations from each pair could be scored for both mating type and serotype. The first step in the analysis was to determine whether the serotype frequencies varied in the different *mt* genotypes. A chi-square test failed to reveal significant differences between serotype frequencies in the *mt* genotypes (P = 0.05–0.10), even though the $mt^D/mt^D$ genotype had a somewhat higher frequency of Hd serotypes than the other two classes. Since no striking differences were found in serotype frequencies among the *mt* genotypes and since an analysis of distributions among individual *mt* genotypes was complicated by the large numbers of classes and the relatively small sizes of the samples, the total distributions of mating types and serotypes (Table 2) were tabulated and analyzed. A chi-square test for homogeneity on this distribution revealed no obvious preferential association of the *H* and *mt* phenotypes (P =0.5–0.7).

*Secondary interrelations:* The two analyses just reported appear to eliminate any direct relationship between the H serotype and the mating type systems; i.e., the *H* and *mt* loci are not the same locus, indeed they are not even closely linked, and subnuclear differentiations in the two systems appear not to be correlated. One final question remains: do factors on the H chromosome appreciably influence the course of mating type differentiation? The converse of this question, concerning the influence of the *mt* chromosomes on serotype differentiation, has already been asked in connection with the analysis of phenotype distributions. In that case no clear decision was possible with the evidence available.

In previous studies of mating type determinants (NANNEY, CAUGHEY and TEFANKJIAN 1955; NANNEY 1959), strains differing in mating type *arrays* were found to differ at the *mt* locus; strains differing in mating type *frequencies* were found to differ, in part, in genetic factors elsewhere in the genome. Similar information concerning the differences between Families D1 and B was provided in

TABLE 2

*The distribution of mating types and serotypes among the vegetative progeny of* $H^C/H^D$ *heterozygotes*

| Mating types | Serotypes Hc Found | Hc Exp. | Hcd Found | Hcd Exp. | Hd Found | Hd Exp. | Total |
|---|---|---|---|---|---|---|---|
| I | 23 | 24.2 | 8 | 11.1 | 31 | 26.7 | 62 |
| II | 66 | 56.2 | 21 | 25.6 | 56 | 61.7 | 143 |
| III | 19 | 23.5 | 13 | 10.8 | 28 | 25.8 | 60 |
| IV | 53 | 60.0 | 27 | 27.4 | 73 | 67.0 | 153 |
| V | 7 | 6.3 | 4 | 2.9 | 5 | 6.9 | 16 |
| VI | 43 | 42.8 | 18 | 19.5 | 48 | 47.0 | 109 |
| VII | 9 | 8.3 | 8 | 3.8 | 4 | 9.1 | 21 |
| Selfer | 4 | 3.1 | 3 | 1.4 | 1 | 3.4 | 8 |
| Total | 224 | | 102 | | 246 | | 572 |

TABLE 3

*Mating type frequencies within Family D1, within derived clones of the same* mt *genotype, and in backcrosses to Family D1*

| Crosses | | Mating types I | II | III | V | VI | Total |
|---|---|---|---|---|---|---|---|
| Family D1 | F. 36 | 24 | 92 | 12 | 90 | 254 |
| | E. 53 | 41 | 60.2 | 12.2 | 88 | |
| Backcross | F. 24 | 17 | 24 | 9 | 44 | 118 |
| (4 progeny tests) | E. 24.7 | 19 | 28 | 5.7 | 41 | |
| $F_2$ | F. 38 | 44 | 36 | 13 | 55 | 186 |
| (22 expanded pairs) | E. 38.9 | 30 | 44.2 | 9 | 64.5 | |
| $F_3$ | F. 106 | 72 | 79 | 13 | 148 | 418 |
| (17 progeny tests) | E. 87.4 | 67.4 | 99.4 | 20.1 | 145 | |

this study, and a test of association between the supplementary factors and the *H* locus is possible.

It is first of all possible to demonstrate that the derived $mt^D/mt^D$ homozygotes differ ($P \ll 0.01$) from the original homozygotes (Family D1) in the frequencies of the mating types (Table 3). Generally speaking, the $F_2$ segregants produce more I's and II's and fewer III's than the original Family D1's. These differences are to be ascribed to the segregation of supplementary factors different for the two parental inbreds. Since the $mt^B/mt^B$ homozygotes were not identified, a direct demonstration of the effect of the supplementary factors on this genotype is not possible.

Next one may ask whether mating type frequencies within *mt* genotypes are correlated with *H* genotypes. An affirmative answer would suggest a significant contribution of factors on the *H* chromosome toward the determination of mating type frequencies. For this analysis the $F_2$ data will be ignored; the nine derivatives of a single pair were often alike, suggesting derivation from a single caryonide, and produced "jackpots" of particular mating types which invalidate the usual tests for significance. The $F_3$ and backcross tests can, however, be used in several situations. Comparisons can be made, for example (Table 4), between the mating type frequencies in the $F_3$ progenies of $mt^D/mt^D$ segregants with different *H* genotypes; in the $F_3$ progenies of $mt^B/mt^D$ segregants; and in the backcross tests of the $mt^B/?$ pairs. Other smaller scale tests were available and yielded similar results. In no case was evidence obtained for significant differences in mating type frequencies among the *H* genotypes. This analysis does not exclude the possibility that supplementary factors with slight effects occur on the *H* chromosome, but the determinants of mating type frequencies are not preferentially clustered on this chromosome.

TABLE 4

*The relationship between* H *genotypes and mating type frequencies in progeny tests*

| Series | *H* genotype | Mating type I | II | III | IV | V | VI | VII | Total |
|---|---|---|---|---|---|---|---|---|---|
| $mt^D/mt^D$ | $H^C/H^C$ | 32 | 22 | 26 | 0 | 4 | 38 | 0 | 122 |
| $F_3$ | $H^C/H^D$ | 62 | 34 | 38 | 0 | 7 | 83 | 0 | 224 |
| | $H^D/H^D$ | 12 | 16 | 15 | 0 | 2 | 27 | 0 | 72 |
| | | $\chi^2 = 6.1$ | d.f. $= 7$ | | P $= 0.5 - 0.7$ | | | | |
| $mt^B/mt^D$ | $H^C/H^C$ | 11 | 17 | 18 | 34 | 3 | 23 | 2 | 108 |
| $F_3$ | $H^C/H^D$ | 22 | 33 | 28 | 46 | 4 | 47 | 12 | 192 |
| | $H^D/H^D$ | 8 | 7 | 5 | 1 | 2 | 7 | 0 | 30 |
| | | $\chi^2 = 13.0$ | d.f. $= 8$ | | P $= 0.1 - 0.2$ | | | | |
| $mt^B/?$ | $H^C/H^C$ | 13 | 7 | 12 | 19 | 3 | 30 | 3 | 87 |
| Backcross | $H^C/H^D$ | 25 | 34 | 21 | 27 | 5 | 60 | 0 | 176 |
| | $H^D/H^D$ | 23 | 39 | 21 | 31 | 8 | 43 | 6 | 171 |
| | | $\chi^2 = 15.0$ | d.f. $= 10$ | | P $= 0.1 - 0.2$ | | | | |

## SUMMARY AND CONCLUSIONS

This analysis demonstrates that the H serotype and mating type systems in variety 1 of *T. pyriformis*, in spite of their many similarities, are in fact distinct and independent systems of cellular differentiation. The *H* and *mt* loci are not the same loci; they show no evidence of linkage and are probably located on different chromosomes. Moreover, in genotypes permitting both kinds of differentiation, no correlation is found between the serotypes and mating types of terminal cultures. This observation would indicate that the events responsible for subnuclear differentiations of one kind are independent of the events responsible for the subnuclear differentiations of the other kind. Finally, no marked effects of *H* chromosomal elements on mating type frequencies or of *mt* chromosomal elements on H serotype frequencies have been demonstrated.

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# THREONINE, ISOLEUCINE, AND ISOLEUCINE-VALINE MUTANTS OF SALMONELLA TYPHIMURIUM[1]

E. V. GLANVILLE[2] AND M. DEMEREC[3]

*Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, New York*

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THIS paper reports an analysis, by transduction techniques, of the genetics of a series of mutants of *Salmonella typhimurium* that are blocked in the synthesis of the amino acids threonine, leucine, isoleucine, and valine. The results were summarized earlier in the Carnegie Institution of Washington Year Book (DEMEREC, LAHR, MIYAKE, GOLDMAN, BALBINDER, BANIČ, HASHIMOTO, GLANVILLE, and GROSS 1958).

The pathway of biosynthesis of these amino acids has been described in several organisms, and summarized by KNOX and BEHRMAN (1959). The diagram of the pathway shown in Figure 1 indicates the probable points in the synthetic sequence at which the different mutant groups described in this paper are deficient. The approximate positions of the blocks were determined primarily on the basis of growth-requirement and feeding tests, and also by analogy with similar mutants, in other organisms, whose enzymatic properties are known. We have not attempted enzymatic analysis of the mutants studied here; and therefore the precise position of the block in each group is not definite, except in the case of the isoleucine-valine mutants. These have been analyzed biochemically by DR. R. P. WAGNER, whose results are reported in the paper following this one (WAGNER and BERGQUIST 1960).

Table 1 lists the mutants in each group, classified according to their approximate spontaneous mutability. Table 2 shows the growth responses of a representative mutant from each group, and Table 3 gives the results of feeding tests between the groups.

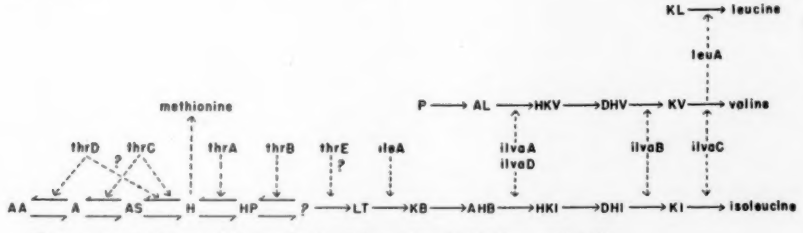


FIGURE 1.—Pathway of biosynthesis of threonine, isoleucine, and valine.

[1] Aided by a grant from the American Cancer Society, Inc.

[2] Present address: Department of Genetics, Trinity College, Dublin, Ireland.

[3] Present address: Department of Biology, Brookhaven National Laboratory, Upton, New York.

TABLE 1

*Classification of the mutants studied, according to group (locus) and degree of spontaneous mutability*

| Locus | No. mutants | Mutability | | |
|---|---|---|---|---|
| | | Low | Intermediate | High |
| *thrA* | 14 | *thr-2,* 8, 9, 10, 59* | *thr-21, 27, 30, 34, 36, 38, 39, 40, 50* | |
| *thrB* | 16 | *thr-12, 13, 23, 29, 49* | *thr-17, 18, 22, 24, 31, 32, 33, 34, 35, 43* | *thr-28* |
| *thrC* | 5 | *thr-11** | | *thr-5, 7, 14, 15* |
| *thrD* | 1 | | | *thr-16* |
| *thrE* | 1 | *thr-60* | | |
| *ileA* | 12 | *ile-3, 6, 11, 13, 14, 16, 18, 23, 24* | *ile-19* | *ile-15, 17* |
| *ilvaA* | 6 | *ilva-8, 12, 14, 19, 22* | | *ilva-11* |
| *ilvaB* | 7 | *ilva-2,* 6, 7, 9, 10, 15, 18* | | |
| *ilvaC* | 2 | *ilva-13, 16* | | |
| *ilvaD* | 1 | *ilva-27* | | |
| *leuA* | 107 | | | |
| Total | 172 | | | |

* Indicates high rate of mutation of a suppressor gene, producing slow-growing "revertants".

TABLE 2

*Growth responses of mutants*

| Mutant | L-homoserine | L-threonine | D-threonine | isoleucine | KB | AIB | AL | valine | DH | DHV | DH + DHV | KI | KV | KV + KI | isoleucine + valine | KL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *thrD-16* | + | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| *thrC-11* | + | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| *thrA-10* | — | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| *thrB-13* | — | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| *thrE-60* | — | + | — | + | + | + | — | — | + | — | + | + | — | + | + | — |
| *ileA-14* | — | — | ± | + | + | + | — | — | + | — | + | + | — | + | + | — |
| *ilvaD-27* | — | — | — | — | — | — | — | ± | — | ± | — | — | ± | + | + | — |
| *ilvaA-8* | — | — | — | — | — | — | — | — | — | — | — | — | — | + | + | — |
| *ilvaB-10* | — | — | — | — | — | — | — | — | — | — | — | — | — | + | + | — |
| *ilvaC-13* | — | — | — | — | — | — | — | — | — | — | — | — | — | — | + | — |
| *leuA-39* | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | + |

+ = good growth response.
± = slight growth response.

TABLE 3

*Feeding relationships*

| Mutants feeding | *thrD-16* | *thrC-11* | *thrA-10* | *thrB-13* | *thrE-60* | *ileA-14* | *ilvaD-27* | *ilvaA-8* | *ilvaB-10* | *ilvaC-13* | *leuA-39* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *thrD-16* | — | — | — | — | — | — | — | — | — | — | — |
| *thrC-11* | — | — | — | — | — | — | — | — | — | — | — |
| *thrA-10* | + | + | — | — | — | — | — | — | — | — | — |
| *thrB-13* | — | — | — | — | — | — | — | — | — | — | — |
| *thrE-60* | — | — | — | — | — | — | — | — | — | — | — |
| *ileA-14* | — | — | — | — | + | — | — | — | — | — | — |
| *ilvaD-27* | — | — | — | — | + | + | — | — | — | — | — |
| *ilvaA-8* | — | — | — | — | + | + | — | — | — | — | — |
| *ilvaB-10* | — | — | — | — | + | + | — | — | — | — | — |
| *ilvaC-13* | — | — | — | — | + | + | + | + | + | — | + |
| *leuA-39* | — | — | — | — | ± | — | + | ± | ± | — | — |
| wild type | — | — | — | — | — | — | — | — | — | ± | — |

+ = good growth response.
± = slight growth response.

## MATERIAL AND METHODS

The mutants were from a collection accumulated by several members of this laboratory in work with strains LT-2 and LT-7 of *S. typhimurium* obtained from Dr. N. D. Zinder. Most of them were of spontaneous origin; a few had been isolated from bacteria treated with ultraviolet radiation. Phage stocks were derived from the temperate form (H-1) of PLT-22 (Zinder and Lederberg 1952).

We used Davis's minimal medium (Lederberg 1950). In transduction experiments it was supplemented with various amino acids, at a concentration of 20 μg/ml. For syntrophism experiments it was enriched with 0.01 percent nutrient broth powder; and in complementation experiments it was unsupplemented.

Transduction was effected by mixing recipient bacteria from a saturated broth culture ($2 \times 10^9$ cells/ml) with phage, at a multiplicity of infection of five. Adsorption was allowed to proceed at room temperature for five minutes before the cells were plated on selective medium. Plates were incubated for 48 hours at 37°C. When necessary, the genetic constitution of transductant colonies was determined by the replica-plating technique. When a large number of similar mutants was to be tested by transduction, as in the *leu* group, it was done by the spot-testing method. An aliquot of a bacterial culture (about $2 \times 10^8$ cells) was spread on a plate containing enriched minimal medium, and drops of different phage suspensions (about $5 \times 10^8$ particles) were added at four or more marked spots. Recombination between recipient and donors could be observed on the areas of the spots.

Feeding between strains (syntrophism) was tested by streaking saline suspensions of the two mutants at right angles on enriched minimal agar, and incubating for 48 hours at 37°C.

Growth requirement was determined either by streaking a saline suspension of cells on enriched minimal agar containing the growth factor in a concentration

of 20 μg/ml, or by spreading it on enriched minimal agar and adding crystals or a drop of the growth factor. In the latter procedure, a halo of growth around the spot signified a positive result.

*Symbols and abbreviations:* The symbols for the kinds of mutants discussed in the paper are as follows: *ara⁻*, inability to utilize arabinose; *arg*, arginine requirement; *asa*, aspartic acid requirement; *azi-r*, azide resistance; *chl-r*, chloromycetin resistance; *cys*, cystine requirement; *gly*, glycine requirement; *ile*, isoleucine requirement; *ilva*, requirement for isoleucine plus valine; *leu*, leucine requirement; *lys*, lysine requirement; *met*, methionine requirement; *nic*, nicotinic acid requirement; *pan*, pantothenic acid requirement; *phl*, phenylalanine requirement; *pro*, proline requirement; *ser*, requirement for serine or serine-glycine alternative; *str-r*, streptomycin resistance; *thi*, thiamine requirement; *thr*, threonine requirement; *try*, tryptophan requirement.

The complete symbol for an individual mutant marker includes a letter designating its locus and a numeral identifying it as an independently derived mutant. Examples: *serB-80*, *thrA-59*.

Biochemical compounds are represented in Figure 1 and elsewhere by the following abbreviations: A = β-aspartyl phosphate; AA = aspartic acid; AHB = α-aceto-α-hydroxybutyric acid; AL = α-acetolactic acid; AS = aspartic-β-semialdehyde; DHI = α, β-dihydroxy-β-methylvaleric acid; DHV = α, β-dihydroxyisovaleric acid; H = homoserine; HKI = α-keto-β-hydroxy-β-methylvaleric acid; HKV = α-keto-β-hydroxyisovaleric acid; HP = homoserine phosphate; KB = α-ketobutyric acid; KI = α-keto-β-methylvaleric acid (ketoisoleucine); KV = α-ketoisovaleric acid (ketovaline); LT = L-threonine; P = pyruvic acid.

*Complementation and abortive transduction:* Mutants were divided into groups primarily on the basis of genetic complementation, which was determined by the occurrence of abortive transduction between members of different groups but not within a group (Ozeki 1956; Demerec and Ozeki 1959). Abortive transduction gives rise to minute colonies. In stable transductants, which form a large colony, there is permanent recombination between the donor and recipient genomes, but in the progeny of an abortively transduced cell the genetic fragment derived from the donor strain does not multiply and does not recombine with the recipient genome.

*Linkage relations and the order of gene loci:* Two markers can be simultaneously transduced only if their loci are carried in the same transduction fragment; that is, they must be closely linked. Thus the occurrence of simultaneous transduction is the best criterion of such linkage and was so employed in this work whenever possible. The order of loci within a transducing fragment was determined in two ways:

1. By finding the ratio of the number of joint incorporations of two donor markers to the number of recombinations between them and the recipient markers, which is an indication of the distance between the two loci on a linkage map. Tables 6, 7, 9, 10, and 11 give the results of transduction experiments between one standard marker as donor (or recipient) and a series of other markers.

The ratio of joint to single transductions decreases with increase in the distance between the loci.

2. By application of the three-point test of classical genetics (DEMEREC and HARTMAN 1956). When the double mutant *serB-80 thrA-59* is transduced with phage from the wild type and plated on minimal medium supplemented with serine, which is selective for *thr*$^+$ transductants but not for the *ser* marker, and when the genotypes of the transductants are later determined by replica plating on medium lacking serine, a characteristic proportion of the *thr*$^+$ cells is also found to be *ser*$^+$. If the donor is not the wild type but another *thr* mutant (e.g., *thrB-13*), there is a distortion in the frequency of *ser*$^+$ transductants, depending on the position of *thrB-13* in relation to the *serB-80* and *thrA-59* markers. Table 7 gives the results of these and of reciprocal tests. The possible relative positions of the three markers are shown in Figure 2; the regions where recombination can occur are numbered. Table 4 shows the locations of the crossovers that would be necessary to produce *thr*$^+$ *ser*$^+$ recombinants in the reciprocal crosses, for each of the three possible orders of loci, together with the observed frequencies of *thr*$^+$ *ser*$^+$ transductants. In accordance with these results, Order II can be excluded. We can exclude Order III on the basis of results of two-point tests (Table 5), which show that frequency of recombination between *thrB-13* and *thrA-59* is very much less than that between *thrB-13* and *serB-80*, and thus, that the two *thr* markers are much closer to each other than to *ser*.

From the results of a series of such tests it is possible to construct a linkage map of markers located within one transducing fragment. A comparison of the efficiency of the two methods for determining the order of mutant loci can be made from Tables 6 and 7.

*Ghost colonies after transduction:* In tests involving transduction of *thrA* mutants with phage from *thrC* or *D* mutants, three sizes of colonies appear on the

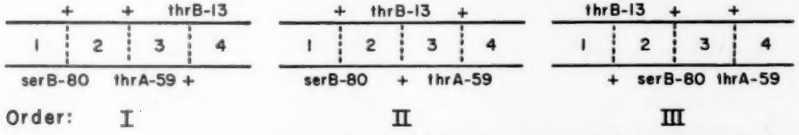


FIGURE 2.—Diagrammatic representation of the three possible orders of the *serB-80*, *thrA-59*, and *thrB-13* markers. The regions where recombination can occur are numbered.

TABLE 4

*Location of the crossovers that would be necessary to produce* thr$^+$ ser$^+$ *recombinants in the reciprocal experiments, for each of the three possible orders of loci*

| Experiment | Order | | | Percent observed |
|---|---|---|---|---|
| | I | II | III | |
| *serB-80 thrA-59* (×) *thrB-13* | 1,3 | 1,2,3,4 | 2,4 | 23.2 |
| *thrB-13* (×) *serB-80 thrA-59* | 3,4 | 2,3 | 1,2 | 96.1 |
| *serB-80 thrA-59* (×) wild | 1,3 or 1,4 | 1,4 | 2,4 | 7.7 |

TABLE 5

*Frequency of recombination (stable transduction), and presence (+) or absence (—) of complementation (abortive transduction), between threonine mutants. Total number of colonies on three plates in experiments made under standard conditions*

| | Donor | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *thrA* | | | | *thrB* | | *thrC* | *thrD* | *thrE* | *serB* | |
| Recipient | Wild | *-8* | *-9* | *-10* | *-59* | *-12* | *-13* | *-11* | *-16* | *-60* | *-80* | Control |
| *thrA-8* | 184 | 0 | 1 | 6 | 15 | 34 | 17 | 38 | 35 | 264 | 146 | 0 |
| | + | — | — | — | — | + | + | + | + | + | + | |
| *thrA-9* | 191 | 0 | 0 | 1 | 4 | 13 | 42 | 27 | 46 | 217 | 206 | 0 |
| | + | — | — | — | — | + | + | + | + | + | + | |
| *thrA-10* | 201 | 0 | 9 | 0 | 3 | 26 | 26 | 29 | 45 | 185 | 177 | 1 |
| | + | — | — | — | — | + | + | + | + | + | + | |
| *thrA-59* | 263 | 4 | 3 | 8 | 0 | 18 | 14 | 45 | 68 | 236 | 269 | 0 |
| | + | — | — | — | — | + | + | + | + | + | + | |
| *thrB-12* | 185 | 6 | 21 | 2 | 8 | 2 | 8 | 28 | 12 | 128 | 129 | 1 |
| | + | + | + | + | + | — | — | + | + | + | + | |
| *thrB-13* | 225 | 23 | 38 | 16 | 6 | 10 | 2 | 66 | 54 | 319 | 204 | 4 |
| | + | + | + | + | + | — | — | + | + | + | + | |
| *thrC-11* | 218 | 46 | 65 | 41 | 35 | 85 | 48 | 1 | 28 | 266 | 211 | 0 |
| | + | + | + | + | + | + | + | — | + | + | + | |
| *thrD-16* | 332 | 210 | 168 | 177 | 89 | 77 | 102 | 58 | 54 | 194 | 186 | 85 |
| | + | + | + | + | + | + | + | + | — | + | + | |
| *thrE-60* | 204 | 171 | 199 | 254 | 142 | 163 | 281 | 254 | 233 | 0 | 181 | 2 |
| | + | + | + | + | + | + | + | + | + | — | + | |
| *serB-80* | 241 | 129 | 256 | 194 | 286 | 199 | 204 | 170 | 188 | 225 | 1 | 2 |
| | + | + | + | + | + | + | + | + | + | + | — | |

plate after 48 hours of incubation: large, minute, and intermediate. Large colonies contain wild-type cells. Minute colonies result from abortive transduction. The intermediate-sized colonies develop from cells of the donor genotype, produced by double transduction of the two markers. They have been called "ghost" colonies because their development is due to feeding by the cells in the background growth of the recipient bacteria. They were first identified in experiments with *try* mutants in 1955 (unpublished observations).

## EXPERIMENTAL RESULTS

*Leucine mutants:* The 107 leucine-requiring mutants analyzed in this study form a single group. They are closely linked genetically, as judged by the low recombination frequencies observed in transduction experiments. From recombination values obtained in reciprocal crosses between different *leu* markers, a linkage map of the *leu* alleles has been constructed, comprising more than 50 sites. Since it is well known that various factors, not yet controllable, may affect frequency of recombination, our present map only indicates the approximate order of the sites within what may be a single locus. All the *leu* markers that we have tested have been found to be closely linked to two loci controlling arabinose

TABLE 6

*Frequency of joint transduction of* ser *and* thr *markers. Selection was made for* thr$^+$ *on minimal-plus-serine plates; and the transductants (either* thr$^+$ ser *or* thr$^+$ ser$^+$*) were replica plated on minimal medium to identify the two classes. Order:* serB—thrD—C—A—B

| Recipient | Donor | Total transductants | No. *ser*$^+$ | No. *ser* | Percent *ser* |
|---|---|---|---|---|---|
| *serB-10* | *thrD-16* | 320 | 213 | 107 | 33.4 |
| | *C-11* | 325 | 227 | 98 | 30.2 |
| | *C-5* | 346 | 250 | 96 | 27.7 |
| | *A-10* | 280 | 229 | 51 | 18.2 |
| | *A-8* | 387 | 323 | 64 | 16.5 |
| | *B-12* | 167 | 145 | 22 | 13.2 |
| | *B-13* | 328 | 293 | 35 | 11.7 |
| | *E-60* | 256 | 256 | 0 | 0.0 |
| *serB-46* | *thrD-16* | 226 | 156 | 70 | 31.1 |
| | *C-11* | 599 | 435 | 164 | 27.3 |
| | *A-10* | 352 | 269 | 83 | 23.5 |
| | *B-12* | 139 | 114 | 25 | 18.0 |
| | *E-60* | 244 | 244 | 0 | 0.0 |
| *serA-11* | *thrD-16* | 210 | 210 | 0 | 0.0 |
| | *C-11* | 349 | 349 | 0 | 0.0 |
| | *B-12* | 162 | 162 | 0 | 0.0 |
| | *A-10* | 96 | 96 | 0 | 0.0 |
| | *E-60* | 338 | 338 | 0 | 0.0 |
| *serB-80 thrA-59* | wild | 141 | 27 | 114 | 19.2 |
| *serB-103 thrC-11* | wild | 247 | 71 | 176 | 28.7 |

TABLE 7

*Order of threonine loci, determined by three-point tests with* serB *and* thr *markers. Selection was made for* thr$^+$ *on minimal-plus-glycine plates (glycine satisfies the requirement in* serB-80 *and* serB-103 *mutants). The transductants (either* thr$^+$ ser *or* thr$^+$ ser$^+$*) were replica plated on minimal medium to identify the two classes. Order:* serB—thrD—C—A—B

| Recipient | Donor | Total | Percent *ser*$^+$ | Percent *ser* |
|---|---|---|---|---|
| *serB-80 thrA-59* | wild | 52 | 7.7 | 92.3 |
| *serB-80 thrA-59* | *thrA-10* | 58 | 6.9 | 93.1 |
| reciprocal | | 162 | 40.7 | 59.3 |
| *serB-80 thrA-59* | *thrB-13* | 86 | 23.2 | 76.8 |
| reciprocal | | 103 | 96.1 | 3.9 |
| *serB-80 thrA-59* | *thrC-11* | 40 | 4.7 | 95.3 |
| reciprocal | | 106 | 37.7 | 62.3 |
| *serB-80 thrA-59* | *thrD-16* | 68 | 5.9 | 94.1 |
| *serB-103 thrC-11* | wild | 130 | 31.5 | 68.5 |
| *serB-103 thrC-11* | *thrA-10* | 140 | 46.4 | 53.6 |
| reciprocal | | 97 | 94.8 | 5.2 |
| *serB-103 thrC-11* | *thrB-13* | 66 | 53.0 | 47.0 |
| reciprocal | | 87 | 96.5 | 3.5 |
| *serB-103 thrC-11* | *thrD-16* | 121 | 15.8 | 84.2 |

fermentation (*araA* and *araB*), and so material is now available for making three-point tests to determine exactly the order of the sites. The leucine mutants are being used by Dr. Paul Margolin in studies of the structure of the *leu* region and its response to mutagens (Margolin, Vandermeulen-Cocito, and Simrell 1959).

Our experiments have shown that *leu* mutants grow on leucine or ketoleucine, but not on ketovaline, their precursor in *Escherichia coli* and probably also in Salmonella (Table 2). In other words, these mutants cannot convert ketovaline to ketoleucine. It has been found that *ilvaC* mutants feed *leu* mutants and presumably accumulate ketoleucine.

In an effort to find out whether any of the *leu* mutants are identical, 52 of them were tested in 572 transduction experiments. In every test recombination was detected, an indication that the mutational change responsible for each one of these 52 mutants had occurred at a different site of the *leu* locus. All 107 mutants investigated are capable of reverting to wild type; therefore none is a multisite mutant.

*Threonine mutants:* Teas, Horowitz, and Fling (1948) described mutants of *Neurospora crassa* that require for their growth either homoserine or methionine plus threonine. They concluded that both methionine and threonine are derived from homoserine. Teas (1950) came to the same conclusion in studying mutants of *Bacillus subtilis*.

In *E. coli*, mutants described by Cohen and Hirsch (1954) have requirements for: (1) L-threonine, (2) L-threonine or L-homoserine, (3) L-threonine plus methionine, (4) L-threonine plus methionine or L-homoserine. The *S. typhimurium* mutants of the present study correspond to the first two of these classes, with the addition of a group growing on either L-threonine or L-isoleucine. Mutants requiring both methionine and threonine have not been detected in Salmonella.

The mutants growing on L-threonine were divided into five groups on the basis of complementation tests. Table 5 records the occurrence of abortive transduction between some of the mutants. Abortive colonies were about ten times more frequent than large colonies. They were clear and distinct in the tests with members of groups *A* and *B* as recipients, less so in the tests of *C*, *D*, and *E* mutants.

The results of growth-requirement tests are summarized in Table 2. Members of groups *C* and *D* grow rapidly on L-threonine and slowly on L-homoserine. Growth on homoserine is greatly stimulated by the addition of isoleucine, but isoleucine alone does not support growth of these mutants. *A*, *B*, and *E* mutants will grow on L-threonine; homoserine does not support their growth. *E* mutants differ from *A* and *B* in being able to utilize L-isoleucine. Neither D-threonine nor D-homoserine is able to support growth of *thr* mutants. Thus *thrA* and *B* mutants are blocked at a later step in the biochemical sequence than *thrC* or *D* mutants; and *thrE* mutants appear to be blocked at a later stage than any of the other *thr* mutants.

The results of syntrophism tests are shown in Table 3. The only feeding ob-

served among the *thr* mutants was by *thrA*, which feeds *C* and *D* but not *B* or *E; thrE* is fed by *ileA* and *ilvaA, B, C,* and *D*.

Two independent lines of evidence indicate that *thrA, B, C,* and *D,* but not *thrE,* are carried in the same donor fragment and, therefore, are closely linked in the bacterial genome.

The yield of prototrophic recombinants in crosses between mutants of *thrA, B, C,* and *D* is always smaller than that in crosses between any of them and wild type (Table 5). The yield of recombinants in crosses between *thrE* and other *thr* mutants approximates that obtained with a wild-type donor, showing that *thrE* is not closely linked to the other *thr* loci.

The four loci *thrA, B, C,* and *D* are closely linked to *serB,* whereas *thrE* is not. Frequencies of joint transduction are shown in Table 6. The results indicate that the order of loci in the transducing fragment is *serB—thrD—thrC—thrA—thrB.* This conclusion was confirmed by three-point tests, made by the method already described, with double mutants carrying both *ser* and *thr* markers (Table 7).

*Isoleucine and isoleucine-and-valine mutants:* The precursors of valine and leucine differ from those of isoleucine (Figure 1), but the enzymes involved in part of their synthesis are the same, and so it might be expected that a single mutation would result in a double or triple requirement. In fact, mutations affecting the enzyme that controls the final step of synthesis of all three amino acids result in an absolute requirement only for isoleucine and a partial requirement for valine. Presumably, alternative transaminases provide for leucine and partial valine synthesis from their keto acids, as has been shown to occur in *E. coli* (RUDMAN and MEISTER 1953).

All the isoleucine-requiring (*ile*) mutants tested fell into a single group. Complementation tests were difficult to interpret owing to the heavy background growth of all mutants on minimal agar. Growth in the absence of isoleucine may be accounted for by the activity of alternative threonine deaminases (UMBARGER and BROWN 1957). All mutants grow on KB, AHB, DHI, KI, and isoleucine. L-threonine has no effect, but D-threonine supports slow growth. Mutants of this group probably lack the "biosynthetic L-threonine deaminase" (UMBARGER and BROWN 1957). The mutants that require both isoleucine and valine or their precursors for growth fall into four groups on the basis of complementation tests (Table 8) and growth requirements (Table 2). Mutants of group *ilvaC* grow only on the amino acids; the corresponding keto acids support growth of *ilvaA, B,* and *D*. According to the sequence of their biochemical blocks, therefore, the order of the *ilva* mutants is: *ilva(A,D)—ilvaB—ilvaC.*

Some groups can also be distinguished by feeding tests (Table 3). Mutants of the *ilvaC* group feed *ilvaA, B,* and *D,* but there is no feeding between members of the last three groups. All four *ilva* groups feed *ileA* and *thrE* mutants. According to feeding tests the sequence of the loci appears to be *(ileA, thrE)—ilva(A,B, D)—ilvaC.*

The growth requirements of the groups, combined with their feeding properties, allow their relative positions in the biochemical sequence to be determined

TABLE 8

*Frequency of recombination (stable tranduction), and presence (+) or absence (—) of complementation (abortive transduction), between* ilva *and* ile *mutants. Total number of colonies on three plates*

| | Donor | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *ilva* | | | | | | | | | *ile* | | *thr* |
| Recipient | Wild | *C-13* | *C-16* | *B-10* | *B-9* | *B-7* | *A-8* | *A-14* | *A-19* | *D-27* | *A-14* | *A-18* | *E-60* | Control |
| *ilvaC-13* | 264 | 1 | 4 | 14 | 47 | 59 | 33 | 68 | 104 | 41 | 76 | 109 | 240 | 1 |
| | + | — | — | + | + | + | + | + | + | + | + | + | + | |
| *ilvaC-16* | 199 | 12 | 1 | 67 | 55 | 94 | 76 | 88 | 142 | 74 | 241 | 89 | 189 | 0 |
| | + | — | — | + | + | + | + | + | + | + | + | + | + | |
| *ilvaB-10* | 342 | 66 | 98 | 0 | 39 | 23 | 48 | 51 | 64 | 49 | 59 | 47 | 245 | 2 |
| | + | + | + | — | — | — | + | + | + | + | + | + | + | |
| *ilvaB-9* | 286 | 18 | 61 | 28 | 0 | 3 | 24 | 32 | 41 | 68 | 72 | 39 | 79 | 0 |
| | + | + | + | — | — | — | + | + | + | + | + | + | + | |
| *ilvaB-7* | 241 | 32 | 59 | 26 | 6 | 0 | 56 | 27 | 39 | 55 | 41 | 78 | 268 | 0 |
| | + | + | + | — | — | — | + | + | + | + | + | + | + | |
| *ilvaA-8* | 309 | 89 | 122 | 42 | 84 | 33 | 1 | 12 | 26 | 89 | 57 | 61 | 175 | 4 |
| | + | + | + | + | + | + | — | — | — | + | + | + | + | |
| *ilvaA-14* | 366 | 122 | 109 | 36 | 64 | 28 | 58 | 1 | 26 | 34 | 73 | 89 | 99 | 0 |
| | + | + | + | + | + | + | — | — | — | + | + | + | + | |
| *ilvaA-19* | 287 | 73 | 27 | 22 | 38 | 49 | 10 | 22 | 0 | 18 | 44 | 51 | 143 | 1 |
| | + | + | + | + | + | + | — | — | — | + | + | + | + | |
| *ilvaD-27* | 182 | 128 | 48 | 55 | 62 | 35 | 28 | 5 | 27 | 0 | 38 | 37 | 254 | 3 |
| | + | + | + | + | + | + | + | + | + | — | + | + | + | |
| *ileA-14* | 332 | 248 | 89 | 77 | 81 | 43 | 42 | 55 | 39 | 41 | 2 | 31 | 171 | 6 |
| | + | + | + | + | + | + | + | + | + | + | — | — | + | |
| *ileA-18* | 285 | 86 | 108 | 64 | 75 | 96 | 72 | 33 | 64 | 36 | 19 | 3 | 244 | 8 |
| | + | + | + | + | + | + | + | + | + | + | — | — | + | |
| *thrE-60* | 251 | 194 | 88 | 87 | 127 | 149 | 48 | 94 | 66 | 127 | 143 | 168 | 2 | 2 |
| | + | + | + | + | + | + | + | + | + | + | + | + | — | |

—except for *ilvaA* in relation to *ilvaD*. The order is: *thrE—ileA—(ilvaA, ilvaD)—ilvaB—ilvaC*.

Detailed studies of the biochemical aspects of the *ilva* mutants have been made by Dr. R. P. Wagner, and are described in this issue (Wagner and Bergquist 1960).

Table 8 gives the results of two-point tests between *ile* and *ilva* mutants, and shows whether or not abortive transduction occurred. The lower frequencies of transduction to prototrophy between members of the different groups than between them and the wild type indicate that *ileA*, *ilvaA*, *B*, *C*, and *D*, but not *thrE*, are closely linked.

The order of the *ile* and *ilva* loci has been investigated by two-point linkage tests (Tables 9, 10, and 11), determining the frequencies of transductant cells that both lost the requirement of the recipient bacteria and acquired that of the donor (double transduction). The higher the frequency of double, compared to single, transduction of the markers, the closer together the loci are presumed to

TABLE 9

*Frequency of joint transduction of* ile *and* ilva *markers. Selection was made for* ilva$^+$ *on minimal-plus-isoleucine plates. Transductants (either* ile$^+$ ilva *or* ile$^+$ ilva$^+$*) were replica plated on minimal medium to identify the two classes. Order:* ileA—ilvaA—D—B—C

| | Donor | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *ile-14* | | *ile-19* | | *ile-18* | | *ile-23* | |
| Recipient | Total | % Donor | Total | % Donor | Total | % Donor | Total | % Donor |
| *ilvaA-7* | 606 | 60.1 | 129 | 63.5 | 99 | 74.7 | 400 | 57.7 |
| *ilvaA-8* | 122 | 67.2 | 165 | 50.9 | ... | ... | ... | ... |
| *ilvaD-27* | 374 | 50.0 | 224 | 50.0 | 131 | 57.3 | 693 | 45.5 |
| *ilvaB-9* | 420 | 41.2 | ... | ... | ... | ... | ... | ... |
| *ilvaB-10* | 267 | 41.6 | 247 | 42.1 | 245 | 39.2 | 238 | 41.2 |
| *ilvaC-13* | 151 | 29.1 | 194 | 18.0 | ... | ... | ... | ... |
| *thrE-60* | ... | ... | 241 | 0.0 | ... | ... | ... | ... |
| *thrB-13* | ... | ... | 304 | 0.0 | ... | ... | ... | ... |
| *thrC-11* | ... | ... | 191 | 0.0 | ... | ... | ... | ... |

TABLE 10

*Frequency of joint transduction of* ilva *markers. Selection was made for the donor type on minimal-plus-KI-plus-KV plates; and transductants were replica plated on minimal medium to identify the two classes. Recipient:* ilvaC-13

| Donor | Total | Percent donor type |
|---|---|---|
| *ilvaA-8* | 53 | 34.5 |
| *ilvaB-12* | 88 | 77.3 |

TABLE 11

*Frequency of joint transduction of* ile *and* ilva *markers. Plating on minimal-plus-valine medium. Donor type appeared as small colonies (ghosts) after three days of incubation at 37°C. Donor:* ilvaD-27. *Order of loci:* ileA—ilvaA—D—B—C

| Recipient | Total | Percent donor | Recipient | Total | Percent donor |
|---|---|---|---|---|---|
| *ileA-14* | 175 | 24.0 | *ilvaC-13* | 106 | 12.5 |
| *ilvaA-7* | 131 | 30.5 | *ilvaC-16* | 133 | 12.0 |
| *ilvaA-8* | 293 | 42.7 | *thrE-60* | 179 | 0 |
| *ilvaB-10* | 82 | 35.4 | *thrB-13* | 298 | 0 |
| *ilvaB-9* | 210 | 26.2 | | | |

be on the linkage map. The absolute frequency of joint transduction varies when different donor strains are used (cf. *ilvaD-27* and *ileA-14*), but all results agree that the order of the loci is *ileA—ilvaA—ilvaD—ilvaB—ilvaC*. The distance between *ileA* and *ilvaA* markers seems to be greater than the distance between other groups of markers in the series.

Although these results are strongly indicative of the order shown above, it cannot be proved conclusively without three-point tests. Attempts to isolate the

double mutant *ile ilva* have failed, and as no other marker is known to be linked to the group, we have not been able to carry out a more rigorous analysis.

## DISCUSSION

The most striking result of this study is the finding that gene loci controlling biochemically related functions are clustered together on the chromosome and are arranged within each cluster in such a way that their order corresponds to the sequence of the biochemical reactions they control. The "assembly line" order exemplified by the four threonine and five isoleucine-and-valine loci has previously been demonstrated with respect to four tryptophan loci (DEMEREC and Z. HARTMAN 1956), eight histidine loci (P. E. HARTMAN 1956; HARTMAN, LOPER, and SERMAN 1960), and very likely three proline and three pantothenate loci (MIYAKE and DEMEREC 1960; DEMEREC, LAHR, BALBINDER, MIYAKE, MACK, MACKAY, and ISHIDSU 1959).

The biosynthetic and genetic order of the loci involved in isoleucine synthesis is:

| *thr(D,C)—thrB—thrA* | *thrE* | *ileA—ilva(A,D)—ilvaB—ilvaC* |

These loci are carried in three separate transducing fragments, as indicated above. The significance of the points of breakage is not clear, and the relative positions of the fragments in the total genome of the cell are not yet known. The question is being investigated by the recently developed technique for studying conjugation between *S. typhimurium* and *E. coli* (MIYAKE and DEMEREC 1959), which makes it possible to analyze the order of markers located in different donor fragments.

A linkage map of each of the loci involved in threonine and isoleucine synthesis is shown in Figure 3. The order of the mutational sites is based on the results of two-factor recombination tests and, thus, is only approximate.

A number of *leu*, *ile*, *ilva*, and *thr* mutants were tested for joint transduction (close linkage) with the following markers: *araA-1; arg-3, -4, -5; asa-3; cysA-20, B-18, C-38, D-23, E-2; gly-2; lys-1; metA-15, B-49, C-50, E-47, F-59; nic-1; pan-2; phl-5; proA-39, B-12, C-96, D-102; serA-11, B-80; thi-1; tryD-7; azi-r* (0.5 $\mu$g); *chl-r* (10 $\mu$g); and *str-r* (100 $\mu$g). Joint transduction was detected only

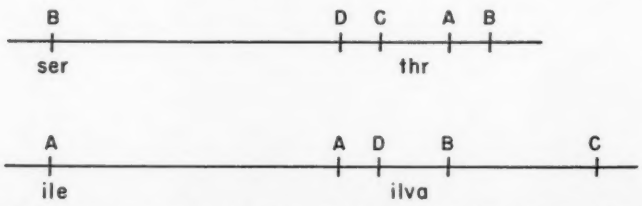


FIGURE 3.—Linkage maps of *ser–thr* and *ile–ilva* fragments. Relative distances between points on the maps correspond approximately to the recombination frequencies. The approximate order of alleles within certain of the loci is as follows: *thrD-7, -5, -11, -15, -14; thrA-10, -8, -9; thrB-17, -12, -13; ileA-19, -11, -14, -3, -6, -2, -16, -18, -17, -15, -13; ilvaA-8, -12, -19, -14, -11; ilvaB-18, -2, -10, -15, -6, -21, -7, -9; ilvaC-13, -16.*

between *leu* and the two arabinose-utilization loci (*araA* and *araB*), and between *serB* and four of the *thr* loci (*thrA, B, C,* and *D*).

Transduction studies with *E. coli* strains K-12 and B (LENNOX 1955; DEMEREC *et al.* 1958) have shown that phage P1 is able to transfer markers arranged in the following order: *thr—ara—leu—pan.* The results (DEMEREC *et al.* 1958) indicate that this whole section cannot be carried by one phage particle but that some particles carry the *thr–leu* and others the *leu–pan* region. Conjugation studies with Salmonella have revealed that the *thr, leu,* and *pan* loci are close together and are arranged in the same order as the functionally similar loci in *E. coli* (DEMEREC *et al.* 1959). Joint transduction of either *thr* and *leu* or *leu* and *pan* markers has not been detected, however, in tests made on a large enough scale to justify the conclusion that any one transducing fragment carried by the Salmonella phage PLT-22 is unable to include two of these three loci.

The difference between the observed behavior in *E. coli* and that in Salmonella might be explained as due to the difference in size of the two transducing phages. Since phage PLT-22 is considerably smaller than phage P1, it seems probable that it can carry less chromosomal material. OZEKI's (1959) results indicate that for any one section of bacterial chromosome transducing fragments carried by phage PLT-22 are the same; that is, the limits of each fragment are predetermined. Thus, it appears probable that the bacterial chromosome has definite weak spots, at which it can break, and that a donor phage has the capacity to transport the fragments located between breaks. In that case it can be supposed that a small phage particle, like that of PLT-22, could carry only a small portion of chromosome—one fragment, resulting from two adjacent breaks—whereas a larger phage, like P1, might carry lager pieces comprising two or more adjoining fragments.

The leucine region, where 107 mutations have been investigated, reveals two interesting characteristics: a lack of sites at which spontaneous changes frequently occur (hot spots), and a lack of structures that would produce multisite changes with the frequency usually observed in Salmonella. As we have mentioned, transduction tests between 52 *leu* mutants showed that every one of the mutations producing them had occurred at a different site of the *leu* locus. If one assumes that the different sites of this gene locus mutate with equal or nearly equal frequency, an estimate can be made of the number of sites present at the *leu* locus. Calculations estimating this number have been made by PROFESSOR HOWARD LEVENE, Department of Mathematical Statistics, Columbia University. His statement regarding "inferences on number of mutational sites" follows:

"Let there be $r$ independently arisen mutations and $n$ mutational sites. If the probability of mutation is the same at each site, then the probability that each of the $r$ mutations is at a different mutational site is $p = \frac{n!}{(n-r)!\, n^r} = \frac{n(n-1)\ldots(n-r+1)}{n^r}$. As an approximation, $\log_e p = -\frac{r(r-1)}{2n}$. (See FELLER, *Probability Theory and Its Applications*, 1st ed. page 29, 2nd ed. page 31, 'birthday problem.')

"Now, to find the minimum number of mutational sites, we find a number of sites, $n$, for which the probability of all $r$ mutations being different is some small number, $\alpha$, say $\alpha = .05$ or $\alpha = .01$. We then must solve

$$log_e \alpha = -\frac{r(r-1)}{2n} \text{ for } n,$$

or

$$n = -\frac{r(r-1)}{2\, log_e \alpha}. \tag{1}$$

"It should be noted that the approximate formula

$$\mathrm{p} = e^{-\frac{r(r-1)}{2n}} \tag{2}$$

is not valid when p is small (that is, when $n$ is small), and the exact formula should then be used:

$$\mathrm{p} = antilog_{10}\,[log_{10} n! - log_{10}(n-r)! - r\, log_{10} n]. \tag{3}$$

*The Biometrika Tables, Statistical Tables and Formulas* by HALD (Wiley), and volume 1 of PEARSON's tables give $log_{10} n!$ up to $n = 1000$. For $n = 55$ the percentage error in p from formula (2) is about seven percent for $n = 1000$ and 11 percent for $n = 500$, whereas the corresponding error in $n$ from formula (1) is two percent for $\mathrm{p} = .217$ and three percent for $\mathrm{p} = .05$.

"Table 12 gives $n$ and p (or $\alpha$) calculated by (3) for $n$ up to 1000 and by (1) for $n > 1000$.

"The important conclusions are: If the number of mutable sites is 230, the probability that all 55 mutations are different is only .001: that is, 230 is the lower 0.1 percent confidence limit of $n$. Similarly, 335 is the lower one percent confidence limit and 510 the lower five percent confidence limit. The most reasonable number of sites is the 50 percent limit, that is, the number $n$ for which there is a 50-50 chance of all mutants' being different; this value is 2100. Finally, the maximum likelihood estimate of $n$ is $\infty$ as long as all mutants are different. This last estimate is clearly unreasonable.

TABLE 12

*p = probability that all of* r = 55 *mutants are different when there are* n *sites of mutation*

| Exact | | Approximate* | |
|---|---|---|---|
| $n$ | p | p | $n$ |
| 200 | .00027 | .001 | 230 |
| 250 | .00162 | .01 | 335 |
| 300 | .00508 | .05 | 510 |
| 400 | .0204 | .10 | 650 |
| 500 | .0457 | .25 | 1070 |
| 646 | .0938 | .50 | 2100 |
| 991 | .217 | .75 | 5200 |
| | | .90 | 14000 |
| | | .95 | 26000 |
| | | .99 | 150000 |

* For $n < 1000$, $n$ obtained by graphic interpolation from exact table; for $n > 1000$, $n$ obtained by formula (1).

"Note that if the mutation rate differs at different sites, all the above values are too small."


## SUMMARY

The 107 leucine-requiring mutants studied form a single group, and the *leu* locus is closely linked to the *araA* and *araB* loci. Intercrosses involving 52 of the mutants showed that all the mutations had occurred at different sites of the locus. On the assumption that the probability of mutation is the same at each site, calculations made by PROFESSOR HOWARD LEVENE estimate the number of possible mutational sites as about 300 at the one percent confidence limit, about 500 at the five percent confidence limit, and about 2000 at the 50 percent confidence limit. These values are too small if the mutation rate differs at different sites.

Mutants requiring L-threonine for growth (*thr* mutants) fall into five groups, distinguishable by complementation. All but two of the groups (*C* and *D*) can also be distinguished by their growth requirements and cross-feeding properties. From these properties the order of the groups with regard to their biosynthetic blocks has been determined. The relative order is: *thr(C,D)—thrA—thrB—thrE*. Four of the loci (*thrA, B, C, D*) are carried in the same transducing fragment as *serB*. The order on a linkage map is *serB—thrD—thrC—thrA—thrB*. Thus the genetic sequence of the loci corresponds to the sequence of the biochemical steps they control. The fifth locus, *thrE*, is not linked to the others.

On the basis of complementation, mutants blocked in the synthesis of isoleucine (*ile*) and isoleucine-valine (*ilva*) have been divided into five groups, representing five gene loci. All five groups can be distinguished by their growth requirements or feeding effects, and from these properties the position of the block in the biochemical sequence in each group of mutants has been inferred. Only the relative positions of *ilvaA* and *D* cannot be discovered by this method. For the rest, the biochemical sequence corresponds to the genetic sequence of the loci: *ileA—ilva(A,D)—ilvaB—ilvaC*.


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# NATURE OF THE GENETIC BLOCKS IN THE ISOLEUCINE-VALINE MUTANTS OF SALMONELLA

R. P. WAGNER AND ARLOA BERGQUIST

*The Genetics Laboratory of the Department of Zoology, The University of Texas, Austin, Texas*

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THE metabolic pathways leading to the biosynthesis of isoleucine and valine are now sufficiently well understood, as a result of the work of a number of investigators (STRASSMAN, THOMAS and WEINHOUSE 1955; STRASSMAN, THOMAS, LOCKE and WEINHOUSE 1956; STRASSMAN, SHATTON, CORSEY and WEINHOUSE 1958; UMBARGER 1958a,b; ADELBERG 1955; WAGNER, RADHAKRISHNAN and SNELL 1958; RADHAKRISHNAN, WAGNER and SNELL 1960; and RADHAKRISHNAN and SNELL 1960) to make it possible to investigate the nature of the genetic blocks in mutant organisms requiring isoleucine and valine. This communication describes the results of the investigation of a series of mutants of *Salmonella typhimurium* originally isolated in the laboratory of DR. M. DEMEREC, The Carnegie Institute of Washington, Cold Spring Harbor, New York. It is limited to the purely biochemical aspects of these mutants, but is preceded by a communication from GLANVILLE and DEMEREC 1960, which describes the linkage studies made with these mutants, and correlates the genetic with the biochemical data.

The biosynthesis of isoleucine and valine is believed to occur as shown in Figure 1. In the work to be described here only the steps proceeding from the $\beta$-keto acids, $\alpha$-acetolactic acid and $\alpha$-aceto-$\beta$-hydroxybutyric acid, have been considered in detail. The following abbreviations are used in Figure 1 and in subsequent parts of this communication: AHB = $\alpha$-aceto-$\alpha$-hydroxybutyric acid;

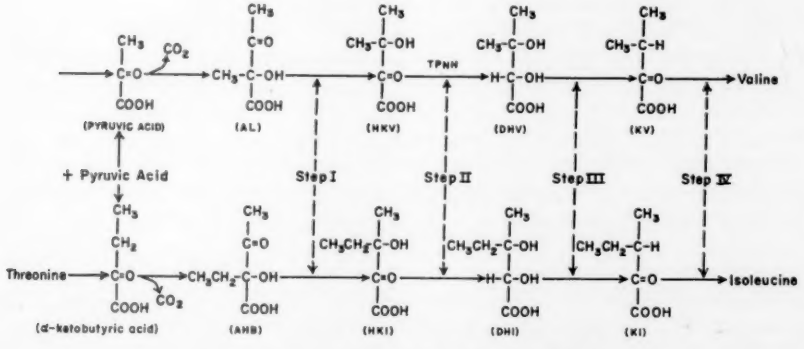


FIGURE 1.—The biosynthetic pathway leading to isoleucine and valine.

AL = α-acetolactic acid; HKI = α-keto-β-hydroxy-β-methylvaleric acid; HKV = α-keto-β-hydroxyisovaleric acid; KI = ketoisoleucine = α-keto-β-methylvaleric acid; KV = ketovaline = α-ketoisovaleric acid.

## METHODS AND MATERIALS

All strains of *Salmonella typhimurium* used in this study were obtained from DR. M. DEMEREC, Carnegie Institute of Washington. The isoleucine plus valine requiring mutants, designated as *ilva*, are grouped into four categories designated as *A*, *B*, *C* and *D*. (See accompanying paper by GLANVILLE and DEMEREC 1960). The wild type strain, LT-2, analyzed for comparative purposes, is the parent strain of several of the mutant strains analyzed.

The minimal medium of DAVIS (LEDERBERG 1950), with the appropriate supplements as indicated, was used in all experiments. All growth tests were carried out at 37°C for the times indicated. Growth attained in broth was determined by measuring turbidity as optical density at a wave length of 600 mμ with a Bausch and Lomb Colorimeter.

Auxonography was done in 20 × 30 cm minimal agar plates. Whatman No. 1 filter paper discs, 1.2 cm in diameter, were placed on the agar surfaces separated by a distance of approximately 1 to 1.5 cm. Twenty to 40 μg of supplement was added to each disc. The plates were prepared by adding cells of a mutant strain in the logarithmic phase of growth to minimal agar maintained at a temperature of 40°C, dispersing the cells, and pouring the seeded agar immediately. The filter paper discs were added when the agar hardened, and the various substances to be tested were pipetted onto the pads. Only one supplement was added to each disc. The plates were incubated at 37°C for 18 to 24 hours and then inspected for growth of subsurface colonies. Growth is detected by this method between any two discs which contain the appropriate, satisfactory supplements. Generally, it is manifested as a thin (1 to 3 mm) zone perpendicular to a line between the centers of the discs. When the ratio of the concentrations of the two compounds required is not critical, growth is diffuse between the two discs.

The identification of carbinols and their quantitative estimation was accomplished by methods described by WAGNER, BERGQUIST and FORREST 1959. Ketovaline and pyruvic acid were determined by chromatography of the 2, 4-dinitrophenylhydrazones (WAGNER and BERGQUIST 1955). Since the dinitrophenylhydrazones of ketovaline and ketoisoleucine have about the same $R_f$'s with the solvents used, the identity of ketovaline was confirmed by determining the X-ray diffraction pattern of its hydrazone (WAGNER and BERGQUIST 1955) and comparing it to a known standard.

Crude extracts for the determination of enzyme activities were prepared from cells 48 hours old which were grown in standing culture on minimal medium supplemented with 10 μg of L-valine and L-isoleucine per ml, unless otherwise indicated. The harvested cells were washed twice with distilled water and sonicated for 30 minutes in 0.1 molar phosphate buffer at *p*H 8.0. The sonicate was centrifuged for 20 minutes at 35,000 rpm in a Spinco Model L Centrifuge. The

pellet was discarded and the supernatant diluted to obtain the appropriate protein concentration with 0.1 molar phosphate buffer at *p*H 8.0. Protein concentrations were determined by the trichloracetic acid precipitation method, and the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). Transamination activity for the phenylalanine-ketovaline and ketoisoleucine reaction was measured by the method described by SEECOF and WAGNER 1959 for Neurospora. The other transamination reactions, not involving phenylalanine were determined qualitatively by chromatography of the incubates after four hours incubation at 37°C. Dehydrase activities were determined by the method described by WAGNER, BERGQUIST and KARP 1958 for Neurospora. Reductase and reductoisomerase enzyme activities were determined by a method given by RADHAKRISHNAN, WAGNER and SNELL 1960.

All enzyme activities are reported as specific activities, $\mu$ moles of substrate converted per hour per mg of protein.

## RESULTS

*Growth stimulation:* The double requirement of the *ilva* mutants makes extensive testing of the eight known precursors of the amino acids in all of the possible combinations, a lengthy and laborious procedure if growth is observed in test tubes. Certain of the mutants are strongly inhibited unless the ratio of the two supplementary compounds is within closely defined limits. Additionally, only one of the precursors is available commercially—the others must be synthesized on a laboratory scale with a low yield. Therefore, the method of testing auxonographically was resorted to. This allows one to test many combinations at once, is economical in its use of compounds, and gives gradients of concentrations of compounds outward from their centers of distribution which increases the likelihood for the proper ratio of two required compounds being obtained.

Figure 2 gives the results from testing mutants in each of the indicated groups with all possible combinations of precursors and amino acids. The *C* mutants, *13* and *16*, grow on isoleucine + valine, but on none of the other combinations. The *B* mutants, *6*, *7*, *9*, *10*, *15* and *18*, grow on the amino acids and the keto acids alone or in combination with the amino acids. The *A* mutants, *8*, *12*, *14*, *18*, *19* and *22*, and *D-27* grow in the presence of one of the dihydroxy acids and an amino acid or keto acid, but not on the dihydroxy acids present together. The hydroxyketo acids are effective in stimulating growth together and in combination with most of the other compounds. AHB, ($\alpha$-hydroxy-$\alpha$-acetobutyrate) is effective only with HKV, DHV or KV in stimulating growth. AL ($\alpha$-acetolactate) seems to be effective only for the *A* mutants, and then only when in combination with HKI.

The combinations which stimulated growth on agar plates were retested in broth using, in general, 20 $\mu$g of supplement per ml. In all but a few, (HKV + AHB, DHV + AHB, for *D* and KV + AHB, HKI + KV, HKI + V for *A*) which are indicated with circles around + or ± in Figure 2, growth was obtained in broth in agreement with auxonographic observations. Those combinations which

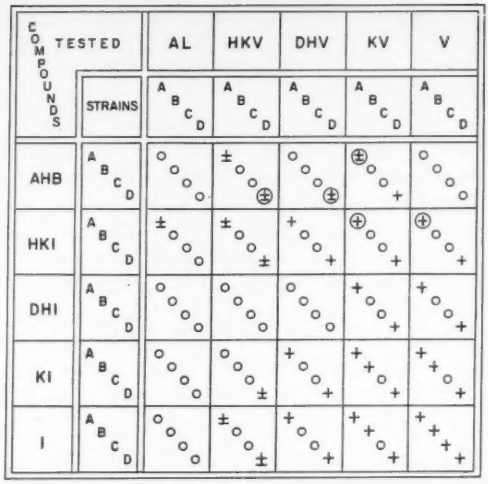


FIGURE 2.—Growth of the members of the *ilvaA, B, C* and *D* groups of Salmonella on different combinations of isoleucine, valine and their precursors. O = no growth stimulation; + = definite growth within 24 hours on broth; ± = slow, but definite growth stimulation. Circled symbols indicate combinations which gave a positive auxonographic test, but were negative in broth.

are designated by ± in Figure 2 gave barely visible growth in broth in about 24 to 30 hours after inoculation whereas those designated with + had completed growth by 30 hours. Approximately the same level of growth was obtained ultimately from all combinations. Minimal controls were run in duplicate or triplicate for all determinations.

The exceptional cases in which bacteria did not grow in broth are understandable, since the compounds were usually tested at only one concentration level. The relative concentrations of the two compounds can be a critical factor as shown in Figure 3. Hence it may be concluded that the auxonographic data are more meaningful than the data from growth in broth when broth tests were negative and auxonography positive.

Strains C-13 and 16 will grow in the presence of isoleucine alone, but not as rapidly as when both isoleucine and valine are present (Figure 4). *D-27* grows on valine alone, but again not as well as when isoleucine is also present (Figure 5).

*Accumulations in the medium:* Three types of compounds may be expected to be accumulated by isoleucine-valine mutants: (1) carbinols, (2) dihydroxy acids and (3) keto acids.

Table 1 indicates the compounds accumulated by the different strains. None of these compounds is produced in detectable amounts by wild type Salmonella. Only qualitative data are given, since the amount of accumulants varies over a

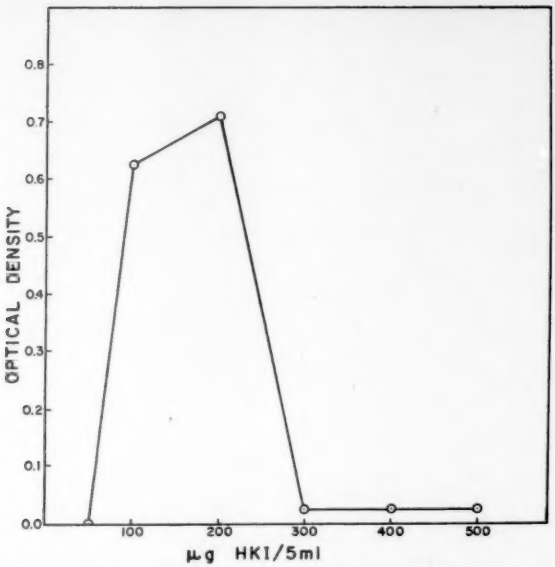


FIGURE 3.—The growth of *ilvaD-27* obtained after 42 hours in broth with 50 μg of valine, and the indicated amounts of hydroxyketoisoleucine per five ml.

TABLE 1

*Compounds accumulated by mutant strains*

| Strain | Carbinols | | Dihydroxy acids | | Keto acids | | PYR | UNK* |
|---|---|---|---|---|---|---|---|---|
| | AMC | AEC | DHV | DHI | KV | KI | | |
| A | + | — | — | — | — | — | + | + |
| B | — | — | + | + | — | — | — | — |
| C | — | — | — | — | + | — | + | + |
| D | + | — | — | — | — | — | + | + |

* Unknown.

wide range depending on the conditions of culture such as initial concentration of supplements, and age of culture.

Only the *B* mutants accumulate detectable amounts of dihydroxy acids. This accumulation is expected from the growth pattern described above. The *A* mutants and *D-27* accumulate carbinol, but only in the form of acetylmethylcarbinol. After 72 hours of growth on medium supplemented with 10 μg of isoleucine and valine the A and D strains accumulate more than 500 μg of AMC per ml of medium. No acetylethylcarbinol was detected after formation of the pteridines.

The accumulation of AMC indicates the preaccumulation of α-acetolactate (see WAGNER, BERGQUIST and FORREST 1959). Acetolactate is readily decarboxy-

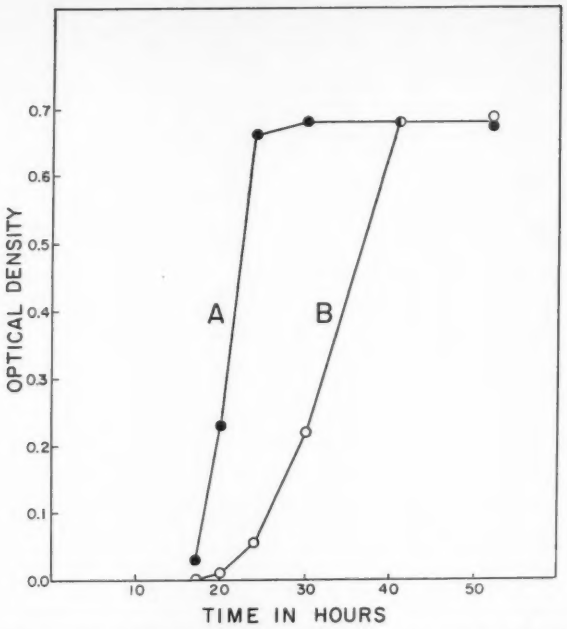


FIGURE 4.—The growth of *ilvaC-13* on isoleucine + valine (A), and on isoleucine alone (B). Amino acids present at levels of 50 $\mu$g/5 ml of medium.

lated with the formation of acetylmethylcarbinol at an acid *p*H or in the presence of an enzyme present in many organisms, (but not tested for here in Salmonella). AL is not unequivocably detectable by chromatographic methods known to us; however, its presence may be at least indicated by oxidation of TPNH in the presence of the appropriate enzyme and the formation of the dihydroxy acid, $\alpha,\beta$-dihydroxyisovaleric acid (DHV). Five ml of medium, 72 hours old, of *D-27* or *A-8* was incubated with 1 ml of a crude enzyme extract of mutant *B-6* containing 3.4 mg of protein, 9.6 $\mu$ moles of glucose-6-phosphate and 1.0 mg. of TPN (triphosphopyridine nucleotide) for 4–6 hours at 35°C. The incubate was then boiled, centrifuged, acidified to *p*H 2 with $H_2SO_4$ and extracted with ether. The ether extracts were chromatogrammed as described elsewhere (WAGNER, RADHAKRISHNAN and SNELL 1958). DHV appeared on the chromatograms when media from *A-8* or *D-27* were present in the incubates but not in the appropriate controls (including *B-6* extract alone). These results indicate the presence of AL in the medium of *A-8* and *D-27*.

The C strains accumulate ketovaline when grown in the presence of isoleucine alone. When valine is present in the growth medium, pyruvic acid appears, and the amount of ketovaline is reduced (Figure 6). Detectable ketoisoleucine is not accumulated by these mutants.

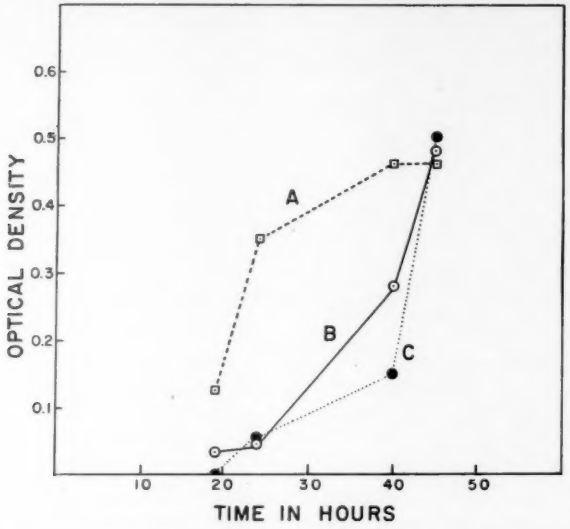


FIGURE 5.—The growth of *ilvaD-27* on valine + isoleucine (A), valine alone (B) and valine + threonine (C). Amino acids present at levels of 50 μg/5 ml.

Pyruvic acid is accumulated by the A strains and D-27.

Treatment of the growth medium of strains of A, D and C with 2, 4-dinitrophenylhydrazine yielded, in addition to the dinitrophenylhydrazone of AMC, KV and pyruvate, a presumed hydrazone of a compound which we have been unable to identify. It has a low solubility in ethyl acetate, water, and ether, and does not move on paper with any of the solvents tried at the same rate as any known dinitrophenylhydrazone. It is not accumulated by strain B or the wild type strain, LT-2.

*Enzymatic activities:* Representatives of each of the four groups of strains were tested for their activity in converting (1) AHB and AL to the respective dihydroxy acids by the over-all enzyme reductoisomerase, in the presence of TPNH and $Mg^{++}$, (2) the hydroxyketo acids to the dihydroxy acids by the reductase in the presence of TPNH, (3) the dihydroxy acids to the keto acids by the dehydrase, and (4) the keto acids to the amino acids by the transaminase. Phenylalanine was used as the donor in the transamination reactions. The results are reported in Table 2 along with activities found for LT-2, a wild type Salmonella.

A-8 and D-27 have enzymatic activities indicated for each of the four steps, but the reductoisomerase activity in each of these is about eight to tenfold lower than LT-2 in activity. Since the figures for these activities, given in Table 2, were determined by measuring the rate of TPNH oxidation in the spectrophotometer, it was necessary to establish whether they represent actual rates of conversion to the dihydroxy acids, or some other type of reaction involving TPNH and the

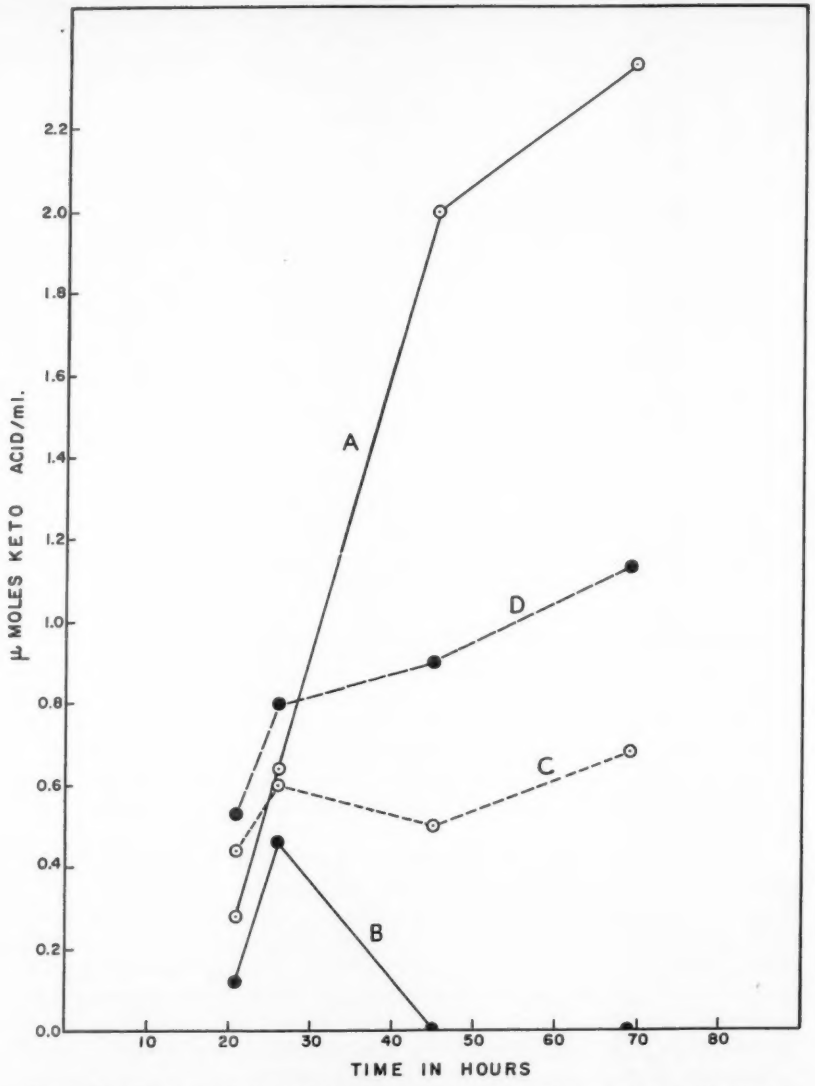


FIGURE 6.—The accumulation of ketovaline and pyruvate by Salmonella *ilvaC-13* with time in broth culture. A. Ketovaline accumulated with growth on 50 μg/5 ml of isoleucine alone. B. pyruvate accumulated under same conditions as in A. C. Ketovaline accumulated with growth on 50 μg of isoleucine plus 50 μg of valine per five ml. D. Pyruvate accumulated under same conditions as in C.

TABLE 2

*Enzymatic activity of the crude extracts of Salmonella strains. Activity given as μ moles of indicated substrate converted per mg protein per hour*

| Strain | Reducto-isomerase | | Reductase | | Dehydrase | | Transaminase | |
|---|---|---|---|---|---|---|---|---|
| | AHB | AL | HKI | HKV | DHI | DHV | KI | KV |
| A-8 | 0.60† | 0.18† | 0.90† | 0.47† | 17.8 | 27.6 | 1.6 | 1.9 |
| B-6 | 50.0 | 5.0 | 6.5 | 3.3 | 0.16 | 0.18 | 1.4 | 1.7 |
| C-13 | 11.0 | 1.1 | 1.4 | 0.70 | 1.9 | 3.4 | 0.0 | 0.0 |
| D-27 | 0.77* | 0.18* | 4.3* | 2.1* | 18.2* | 34.1 | 1.2 | 1.7 |
| LT-2 | 6.0‡ | 1.5‡ | 1.3‡ | 0.80‡ | 0.92‡ | 1.8‡ | 0.50‡ | 0.64‡ |

* 36 hours growth.
† 40 hours growth.
‡ 18 hours growth.

substrates AHB and AL. To test this possibility enzyme extracts of both A-8 and D-27 were incubated with AHB and AL as follows. Two ml of crude, dialyzed extract from 36-hour old cultures containing 2 mg of protein per ml were incubated with 9.6 μ moles of glucose-6-phosphate, 1.0 μ moles of $MgSO_4$, 0.1 mg of TPN, and 0.2 ml of glucose-6-phosphate dehydrogenase (prepared according to the method of RADHAKRISHNAN 1960) and either AL, AHB, HKI, or HKV present at levels of about 10 μ moles each. These mixtures were incubated for six hours at *p*H 8.0 at 35°C. The incubates were then acidified to *p*H 2.0 with $H_2SO_4$ and extracted with ether. The ether extracts were concentrated and chromatogrammed. The papers were sprayed with periodate and benzidine to detect DHI and DHV. Extracts from both D-27 and A-8 showed weak activity for transformation of AHB to DHI and strong activity for HKI and HKV transformation to DHI and DHV, respectively. No DHV was detected when AL was used as the substrate. An extract of B-6 was tested in the same way and found to have all activities including that for conversion of AL to DHV. These results indicate that the weak oxidation of TPNH in the presence of AHB by A-8 and D-27 represents, at least in part, conversion to DHI, but that AL conversion is absent, or so low as to be undetectable. These observations are in agreement with the facts that both mutants apparently accumulate AL, and that D-27 will grow in the presence of valine alone. On the other hand, the rate of conversion of AHB to DHI in A-8 is possibly so low that there is an absolute requirement for isoleucine as well as valine. The absence of detectable AHB or AEC in the medium of these mutants may be due to the utilization of one or both of these in other reactions.

B-6, as shown in Table 2, is significantly lower in dehydrase activity than the wild type and the other strains tested. Strain B-7 has even lower dehydrase activity, and also shows detectable activity for keto acid production from the dihydroxy acids.

C-13 has no detectable activity for transamination between phenylalanine and ketovaline and ketoisoleucine. Extracts from this mutant in addition show no activity for transamination between ketovaline or ketoisoleucine and glutamate or leucine. LT-2, and the other mutants, on the other hand, do have activity for

these transaminations. Hence, it may be assumed that the mutation producing C-13 has caused the absence of an enzyme comparable to the transaminase B described by RUDMAN and MEISTER (1953) in *Escherichia coli*. C-13 does have transaminase A activity which allows it to transaminate between glutamate and the aromatic amino acids, and transaminase C activity enabling it to transaminate between ketovaline and alanine or $\alpha$-aminobutyrate. This latter activity probably explains why C-13 can grow on isoleucine alone. The accumulation of ketovaline under these conditions may be the result of a low activity of the ketovaline-alanine-$\alpha$-aminobutyrate transaminase. These observations make C-13 quite comparable in all respects to a mutant type long known in *Escherichia coli* and described in detail by RUDMAN and MEISTER (1953) and ADELBERG and UMBARGER (1953).

In addition to the evident low relative activity of the reductoisomerase from A and D, the dehydrase from B, and the absence of transaminase activity in *C* mutants, it is also noteworthy that some activities are far in excess of the activity found in the wild type, LT-2, and the mutant *C-13*. Thus B-6 is significantly higher in reductoisomerase activity and reductase than LT-2 and C-13. On the other hand, the dehydrase activity of A-8 and D-7 is 10 to twenty fold higher than LT-2 and almost tenfold higher than C-13. C-13 is close to LT-2 in all specific activities determined with the exception of the phenylalanine transaminase.

## DISCUSSION

From the biochemical analyses presented above it is evident that three different inherited metabolic blocks are represented in the *ilva* mutants.

The *A* mutants and the single *D* mutant are all similar in their low activity of reductoisomerase, and their accumulation of acetylmethylcarbinol and probably acetolactate. All are stimulated by hydroxyketoacids, dihydroxy acids, keto acids and amino acids in certain combinations. There are certain differences, however, since at least one of the *A* mutants (*A-8*) will grow on hydroxyketoisoleucine plus acetolactate (Figure 2). *D-27*, on the other hand, will grow on valine alone which is not true of any of the *A* mutants tested. All evidence taken together indicates that these mutants are all blocked partially or completely at Step I as indicated in Figure 1. All biochemical evidence indicates that a single enzyme acts at this step on both AL and AHB (RADHAKRISHNAN, WAGNER and SNELL 1960). It may be hypothesized that the block is more complete for AL conversion than for AHB conversion, because of the differential affinity of the enzyme for the two substrates.

The *B* mutants are all clearly blocked at Step III, since all accumulate dihydroxy acids, are low in dehydrase activity, and are stimulated to grow only by the keto and amino acids.

The *C* mutants are blocked at Step IV due to the inactivity of the glutamic acid or phenylalanine-ketovaline, ketoisoleucine transaminase. This conclusion is supported by the further evidence that they are stimulated to maximum growth only by isoleucine + valine. Their submaximal growth on isoleucine alone may be

explained by the presence of a ketovaline-alanine or $\alpha$-aminobutyric acid transaminase. The accumulation of ketovaline, but not ketoisoleucine, when the *C* mutants are grown in the absence of valine, is explained by assuming a low activity for the ketovaline-alanine transaminase, and the prevention of ketoisoleucine formation by isoleucine inhibition of the formation of its precursor, AHB (see WAGNER, BERGQUIST and FORREST 1959).

These mutants, then, all fall into three fairly well-defined groups corresponding to blocks at Steps I, III and IV. No mutants have been detected for Step II. This step may, in fact, occur on the surface of the molecule of the reductoisomerase. The relationship of the reductase enzyme to the reductoisomerase is being investigated. It may represent the residual activity of an altered over-all enzyme molecule and be comparable to the tryptophan synthetase enzyme changes described by YANOFSKY (1959) and YANOFSKY and CRAWFORD (1959).

The high enzyme activities found for the reductoisomerase from B-6 and the dehydrase from A-8 and D-27 are probably best explained as being the result of growing the cells in limited amounts of isoleucine and valine. This same general phenomenon has been observed in histidine mutants of Salmonella by AMES, GARRY and HERZENBERG (1960) which were grown in the presence of limiting histidine. It has been explained by AMES and GARRY (1959) in terms of coordinate enzyme repression.

## SUMMARY

Mutants of *Salmonella typhimurium* which are located in three closely linked loci known to be involved in the biosynthesis of isoleucine and valine have been analyzed biochemically. It is shown that each of the three loci controls one of three separate and successive steps in the biosynthesis of these amino acids. The order of the loci, as determined genetically by other workers, corresponds to the order of their action in the sequence of reactions.

## ACKNOWLEDGMENTS

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# LINKED MUTATIONS BORNE BY DEOXYRIBONUCLEIC ACID CONTROLLING THE SYNTHESIS OF CAPSULAR POLYSACCHARIDE IN PNEUMOCOCCUS

ARNOLD W. RAVIN

*Department of Biology, The University of Rochester, Rochester 20, New York*

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AS defined by PONTECORVO (1958), recombination is ". . . any process which gives origin to cells or individuals associating in new ways two or more hereditary determinants in which their ancestors differed . . .". According to this definition, genetic transformations of bacteria constitute a special mode of recombination. In transformation a new combination of hereditary determinants is produced following the exposure of a suitably marked recipient strain of bacteria to the deoxyribonucleic acid (DNA) extracted from a donor strain that differs from the recipient strain by one or more such determinants. Yet, in another sense, transformations are directed mutations, for they confer one or more genetic properties of the donor upon the recipient strain of bacteria. It seems more desirable, however, to treat transformation as a recombination resulting from the confrontation of a portion of the recipient genome with the homologous portion of the donor genome contained in the transforming DNA.

The evidence for this point of view comes from a number of directions. In the first place, it has been demonstrated that, when the recipient cells are physiologically competent, they absorb transforming (donor) DNA from the medium. This has been shown (GOODGAL and HERRIOTT 1957; LERMAN and TOLMACH 1957; FOX 1957) in experiments using transforming DNA uniformly labelled with $P^{32}$. The radioactive phosphorus enters the competent recipient bacteria; the amount that is irreversibly bound is directly proportional to the frequency of transformed bacteria eventually produced; the radioactive label is recoverable specifically from the DNA fraction of the recipient cells. A second piece of evidence is the reversibility of transformation (TAYLOR 1949; HOTCHKISS and MARMUR 1954; RAVIN 1959). A bacterium with genetic property *A* can be transformed into one with the mutated property *a*; using the appropriate DNA, the transformed bacterium can then be transformed back into a bacterium with the property *A*. Such reversibility shows that transformation cannot be regarded as simply the adding on of donor DNA to the DNA already present in the recipient genome, but rather as the replacement of a piece of recipient DNA by a corresponding (homologous) piece contained in the transforming DNA. A third line of evidence is discussed in this paper. Certain transformations are best explained by the assumptions that the recipient and donor bacteria are mutated for a

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certain genetic property at different sites of a specific DNA unit and that transformation combines the unmutated lengths of the recipient and donor units to restore a "wild-type" genome (EPHRUSSI-TAYLOR 1951).

This paper deals with a variety of mutations affecting the ability of the type III strain of Pneumococcus to synthesize the specific polysaccharide that constitutes its extracellular capsule. Two questions are posed: (1) Are these mutations "unlinked", i.e., are they borne by separate transforming DNA particles? (2), If, on the contrary, they are "linked" (i.e., occur at different sites of the same transforming particle), can their sites be mapped with respect to each other?

## MATERIALS AND METHODS

The normal type III capsule-synthesizing strain is designated as SIII-N. Its colonies are smooth (S) in appearance. Mutants deficient in capsule synthesis fall into three more or less distinct phenotypic classes (RAVIN 1959): SIII-2, SIII-1, and R. SIII-2 mutants produce an appreciable amount of capsule. While somewhat smooth, their colonies are nevertheless readily distinguishable from those of SIII-N. SIII-2 colonies are recognizably distinct, moreover, from those of SIII-1 and R mutants.

SIII-1 mutants produce so little capsular polysaccharide that their colonies are rough (R) in appearance. Consequently, while SIII-1 colonies are easily distinguished from those of SIII-2 or SIII-N, they are not distinguishable from those of R mutants, which apparently produce no capsular polysaccharide. Hereafter in this article SIII-1 and R will be referred to simply as capsule-deficient strains. The presence of the small amount of capsule produced by SIII-1 bacteria can be detected by a number of means, and one of these is its interference with anti-R agglutinins. The titer of anti-R agglutinins necessary for complete agglutination is greater for SIII-1 than for R strains (RAVIN 1959).

The heritage of the various mutant stocks has been previously described (RAVIN 1959).

Most of the procedures utilized in these experiments (media, DNA preparation, anti-serum preparation, etc.) have also been previously described (EPHRUSSI-TAYLOR 1951; RAVIN 1959).

In certain experiments discussed below it is necessary to measure the relative frequency of SIII-N and SIII-2 transformants produced in a reaction from which both types may appear. For this measurement a special procedure has been developed, which will be described in the relevant section below.

## EXPERIMENTS

*Linkage of mutations causing impaired capsular synthesis:* In a previous report (RAVIN 1959) an important difference was demonstrated between capsule-deficient strains obtained by spontaneous mutation (hereafter termed "capsule-deficient mutants") and capsule-deficient strains obtained by transformation (hereafter referred to as "capsule-deficient transformants"). The capsule-deficient transformants fail to be converted into encapsulated cells when treated

either with the specific DNA (from a capsule-deficient strain) responsible for their production or with the DNA extracted from cells of their own strain (clone). This finding suggests genotypic identity of the capsule-deficient transformants with the capsule-deficient donor cells. The spontaneous capsule-deficient mutants fail to be transformed into encapsulated cells when treated with DNA extracted from cells of the same strain, but they are generally transformed into encapsulated cells when treated with the DNA extracted from a different (independently-isolated) capsule-deficient strain. This finding demonstrates genotypic differences between the various mutants impaired in type III capsule synthesis.

Two hypotheses can be formulated to account for the genotypic differences of independently-isolated, capsule-deficient mutants (Figure 1). On the one hand, it is possible that many independent (unlinked) factors contribute to capsule synthesis. On the other hand, the different capsule-deficient mutants may be the result of mutations at very closely linked, but recombinable sites within a single region concerned with capsule synthesis. Discrimination between these two hy-

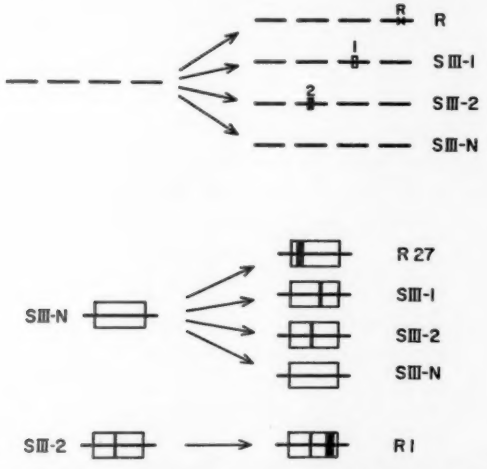


FIGURE 1.—Two alternative schema for representing the sites of mutations impairing the synthesis of capsular polysaccharide. In the upper part of the figure, the different horizontal lines represent separate (unlinked) transforming agents affecting capsule synthesis. As indicated by the vertical bars or symbols, mutations on any one of these agents can cause different degrees of impairment. Replacement of a mutated agent by its normal homologue restores the normal (SIII-N) phenotype. In the lower part of the figure, a single transforming agent is represented as being susceptible of mutations at different sites. The segment of the agent affecting type III capsule synthesis is indicated by the region enclosed by the rectangle. Mutations causing different degrees of impairment are indicated by the width of the segment affected. Recombination between two different mutated agents can restore a totally unmutated segment, which produces the normal phenotype. A transforming agent may bear more than one mutation as indicated in the R1 genotype.

potheses is made possible by the existence of two genetically different strains of type III encapsulated bacteria. The one strain is SIII-N, which produces a greater amount of capsular polysaccharide and consequently larger and more mucoid colonies than the other strain, SIII-2, a mutant derivative. Fortunately, colonies of SIII-2 and of SIII-N cells are morphologically distinct.

Experimental test of these two hypotheses is based on the following facts.

I. Two different capsule-deficient strains, Rx and Rz, arising by mutation from SIII-N interact in the following way:

| Recipient | | DNA donor | | Encapsulated transformants |
|---|---|---|---|---|
| (1) Rx | + | Rx | → | none |
| (2) Rx | + | Rz | → | SIII-N |
| (3) Rz | + | Rx | → | SIII-N |
| (4) Rz | + | Rz | → | none |

II. Two different capsule-deficient strains, R1 and R11, arising by mutation from SIII-2, interact in the following way:

| Recipient | | DNA donor | | Encapsulated transformants |
|---|---|---|---|---|
| (5) R11 | + | R11 | → | none |
| (6) R11 | + | R1 | → | SIII-2 |
| (7) R1 | + | R11 | → | SIII-2 |
| (8) R1 | + | R1 | → | none |

III. A capsule-deficient strain, such as Rx, derived from SIII-N, interacts with the DNA of encapsulated strains in the following way:

| Recipient | | DNA donor | | Encapsulated transformants |
|---|---|---|---|---|
| (9) Rx | + | SIII-N | → | SIII-N only |
| (10) Rx | + | SIII-2 | → | SIII-N + SIII-2. |

IV. A capsule-deficient strain, such as R11, derived from SIII-2, interacts wtih the DNA of encapsulated strains in the following way:

| Recipient | | DNA donor | | Encapsulated transformants |
|---|---|---|---|---|
| (11) R11 | + | SIII-N | → | SIII-2 + SIII-N |
| (12) R11 | + | SIII-2 | → | SIII-2 only |

The two hypotheses mentioned above can account for all of the above facts, but they predict that quite different frequencies of SIII-2 and SIII-N transformants will be produced in reactions 10 and 11. Consider reaction 10:

| | According to: Model of unlinked mutations | | Model of linked mutations | |
|---|---|---|---|---|
| | + | R | + | R |
| the recipient is genotypically: | —\|— | —\|— | —\|——\|— | |
| the DNA donor is genotypically: | —\|— | —\|— | —\|——\|— | |
| | 2 | + | 2 | + |

therefore: the reaction would yield SIII-N transformants frequently and SIII-2 transformants rarely, because, while the former result from single transformation events (the substitution of $\overset{+}{—|—}$ for $\overset{R}{—|—}$), the latter result from double transformations (the substitution of $\overset{2}{—|—}$ for $\overset{+}{—|—}$ and of $\overset{+}{—|—}$ for $\overset{R}{—|—}$). SIII-2 transformants need not be rare; if $\overset{2}{—|—}\overset{+}{—|—}$ substitutes for $\overset{+}{—|—}\overset{R}{—|—}$ as frequently as $\overset{+}{—|—}$ substitutes for $\overset{R}{—|—}$, then SIII-2 transformants will be as frequent as SIII-N transformants.

Finally, consider reaction 11:

| According to: | Model of unlinked mutations | Model of linked mutations |
|---|---|---|
| the recipient is genotypically: | $\overset{2}{—|—}$ $\overset{R}{—|—}$ | $\overset{2}{—|—}\overset{R}{—|—}$ |
| the DNA donor is genotypically: | $\overset{+}{—|—}$ $\overset{+}{—|—}$ | $\overset{+}{—|—}\overset{+}{—|—}$ |

therefore: the reaction would yield SIII-N transformants rarely relative to SIII-2 transformants, because the former result from double transformations. SIII-N transformants need not be rare.

Only one possibility needs to be excluded, however, for the case of reaction 10. It must be demonstrated that $\overset{2}{—|—}$ is not epistatic to $\overset{R}{—|—}$, so that a $\overset{2}{—|—}\ \overset{R}{—|—}$ transformant could be phenotypically SIII-2. Proof that this is not the case is provided by isolating SIII-2 transformants obtained by exposing Rx cells to DNA from SIII-2. The DNA is then extracted from such SIII-2 transformants, and is used to treat more Rx cells. If these SIII-2 transformants were genotypically $\overset{2}{—|—}\ \overset{R}{—|—}$, their DNA could not transform Rx cells (*ex hypothesi*, $\overset{+}{—|—}\ \overset{R}{—|—}$) into SIII-N cells (*ex hypothesi*, $\overset{+}{—|—}\ \overset{+}{—|—}$). Experiments were therefore performed to test the genotype of SIII-2 transformants obtained by reaction 10. This test was performed by extracting the DNA from such transformants, and using it to induce transformations on the same recipient strain as was used in the first transformation. As will be seen from the example given in Table 1, SIII-N transformants were recovered at similar frequencies in the first and second steps

TABLE 1

*Frequency of SIII-N transformants produced in strain Rx by the DNAs of two SIII-2 strains differing in their origin*

| Origin of DNA | Streptomycin-resistant | SIII-2 | SIII-N |
|---|---|---|---|
| SIII-2-str no. 1 | $1.0 \times 10^{-4}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| SIII-2-str no. 2 | $3.4 \times 10^{-4}$ | c. $10^{-3}$ — $10^{-4}$ | c. $10^{-3}$ |

Rx cells were exposed to DNA for 15 hrs. Donor strain no. 1 originally acquired SIII-2 marker by transformation of strain R36A (EPHRUSSI-TAYLOR 1951); streptomycin-resistance (*str*) marker was introduced subsequently by transformation with DNA of strain SIII-1-str. Donor strain no. 2 acquired SIII-2 marker by transformation of strain Rx-str.

of transformation. Therefore, it is concluded that the SIII-2 transformants obtained by reaction 10 are not genotypically $\frac{2}{-|-}$ $\frac{R}{-|-}$.

The technical procedure for measuring the relative frequency of SIII-N and SIII-2 transformants in a reaction from which both types may appear (reactions 10 or 11) is somewhat complicated. The complication is due to the necessity of providing in the reaction mixture antibodies against the untransformed recipient cells; these antibodies increase the ultimate frequency of the transformed encapsulated cells, possibly due to selection (RAVIN 1956, 1959). If there were differential selection for the SIII-N and SIII-2 classes of cells, one could never be sure whether the relatively low frequency of a particular class of transformants were due to the intrinsically low rate of their production by transformation or due to selection against them. To avoid the problem of selection, cells exposed to DNA for a brief period of time (10–20 minutes) are treated with DNase, and then diluted samples are taken to inoculate each of a large number of tubes containing media in which appropriate antibodies are present. The sample size is made so small that the probability of introducing more than one cell contacted by the capsular transforming agent is considerably less than one. After a suitable period of growth ($> 5$ hrs), allowing complete expression of the newly acquired character, the tubes are plated on appropriate media to determine the presence of transformants. (In the case of encapsulated transformants, it is not feasible to obtain an accurate count of their colonies because of an interfering background of a vast majority of untransformed, unencapsulated colonies. In the case of streptomycin- and erythromycin-resistant transformants, however, it is possible to count the number of colonies on antibiotic-containing media by eliminating the vast majority of untransformed sensitive bacteria, and so get a reliable estimate of the frequency of transformants in a given tube.) The proportion of tubes containing SIII-N transformants can be compared to the proportion of tubes containing SIII-2 transformants.

The application of this procedure to the case in which bacteria of strain SIII-1 are treated with the DNA of encapsulated cells is shown in Tables 2 and 3. It should be noted that SIII-1 is a capsule-deficient mutant that was derived from an SIII-N strain and is like strains Rx and Rz in its reactions with the DNA from strain SIII-N and the DNA from strain SIII-2. Table 2 gives the result of an experiment in which SIII-1 cells were treated with the DNA of a strain that

TABLE 2

*Short-term exposure of strain SIII-1 to TP SIII-N-ery$_2$-str*

| Transformant class | No. of tubes examined | No. of tubes containing transformants | | No. of transformants per 0.1 ml* |
|---|---|---|---|---|
| ery-resistant | 50 | 12 | | c. 2500, c. 2000, 1496, 413, 226, 149, 143, 125, 112, 92, 18, 6 |
| str-resistant | 50 | 16 | | c. 3000, c. 3000, 1446, 544, 328, 322, 229, 191, 188, 152, 113, 112, 73, 71, 57, 49 |
| SIII-N | 49 | 10 | | >100 in each tube |
| SIII-2 | 49 | 0 | | |
| | | Observed | Expected† | |
| ery-resist. + str-resist. | 50 | 4 | 3.8 | |
| ery-resist. + SIII-N | 49 | 3 | 2.4 | |
| str-resist. + SIII-N | 49 | 1 | 3.2 | |
| ery-resist. + str-resist. + SIII-N | 49 | 0 | 0.8 | |

* Total no. cells/0.1 ml = $2.3 \times 10^7$.
† On basis of independent transfer of transforming agents affecting different functions.
Grew cells in presence of albumin for 4 hrs 10 min.
Added DNA for 20 min treatment. Added DNase at 4 1/2 hrs, plated samples, and transferred 3300 cells to each of 50 tubes containing 2 ml of medium + anti-R antibodies. Incubated tubes for 14 hrs, and then plated each tube on selective and nonselective media.

TABLE 3

*Short-term exposure of strain SIII-1 to TP SIII-2-str*

| Transformant class | No. of tubes examined | No. of tubes containing transformants | | No. of transformants per 0.1 ml* |
|---|---|---|---|---|
| SIII-2 | 49 | 40 | | >100 in each tube |
| SIII-N | 49 | 20 | | >100 in each tube |
| resistant to 100$\mu$ str/ml (str) | 49 | 31 | | 161, 216, 1295, 227, 214, 80, 6, 34, 75, 102, 128, 1016, 194, 49, 49, 55, 129, 55, 219, 51, 176, 101, 48, 113, 290, c. 2000, 223, 47, 251, 396, 337 |
| | | Observed | Expected† | |
| SIII-2 + SIII-N | 49 | 17 | 16.3 | |
| SIII-2 + str | 49 | 25 | 25.3 | |
| SIII-N + str | 49 | 11 | 12.6 | |
| SIII-2 + SIII-N + str | 49 | 11 | 10.3 | |

* Total no. cells/0.1 ml = $3.2 \times 10^7$.
† On basis of independent transfer of transforming agents affecting different functions.
Similar to experiment described in Table 2.
Time of exposure: 20 min. Number of cells transferred from exposed population to each tube: 7290.

differed in three genetic properties from the recipient strain. The donor DNA contained the "wild-type" determinant for normal capsule synthesis (*SIII-N*), as well as determinants conferring resistance to streptomycin (*str*) and resistance to erythromycin ($ery_2$). In this experiment, the number of tubes containing each of three classes of transformed cells was about the same. No SIII-2 transformants were observed, which conforms to previous results obtained with the reaction of SIII-1 recipients with the DNA of SIII-N donors (EPHRUSSI-TAYLOR 1951; RAVIN 1954). It is interesting, however, that the number of transformants of a given class varies considerably from tube to tube. Thus, for example, some tubes contained as many as 3000 *str* transformants per 0.1 ml while others contained as few as 49 per 0.1 ml. The distribution of the number of transformed cells per tube is quite characteristic for a given cell/DNA reaction system, and has been reproduced in a number of experiments. These results are interpreted in the following way. The fraction of tubes containing transformants of a particular class is determined by the fraction of cells originally exposed to the transforming DNA that absorbed the genetic marker in question. Thus, in the present case, the number of cells absorbing the *str* marker which were inoculated into each tube was 0.38 (given by x in the equation: $e^{-x}$ = proportion of tubes receiving no cells with the *str* marker = 0.68). Therefore, about 26 percent of the tubes received one cell that had absorbed the *str* marker and about five percent of the tubes received two cells that had absorbed this marker. At the time of inoculating the tubes, the lowest frequency of cells containing the *str* marker in a tube containing any at all was one in 3300. Yet the lowest frequency finally observed (at the end of the period of incubation) was 49 in $2.3 \times 10^7$ or one in $4.6 \times 10^5$; the highest frequency was roughly 3000 in $2.3 \times 10^7$ or a little over one in $10^4$. This finding can be taken to mean that not all cells absorbing a given marker integrate it into their genomes during the first generation of growth. A delay in integration of from one to several generations will lower the frequency of genetically transformed cells in the clone descending from the bacterium that originally made effective contact with the DNA determinant. Alternative explanations for the distribution of the number of transformed cells per tube will be discussed below.

Finally, it will be noted that the proportion of tubes containing two or more classes of transformed cells ($ery_2$ + *str* or *str* + *SIII-N*, etc.) is what one would expect if the determinants for the capsule character, the erythromycin-resistance character and the streptomycin-resistance character were absorbed and integrated independently of each other. The expected number of tubes containing both cells transformed for character *a* and cells transformed for character *b* is calculated as the proportion of tubes containing transformants of class *a* multiplied by the proportion of tubes containing transformants of class *b* multiplied by the total number of tubes examined.

Table 3 gives the results of an experiment in which cells of strain SIII-1 were treated in a similar manner with DNA from a streptomycin-resistant SIII-2 strain (i.e., one containing the determinants *str* and *SIII-2*). About twice as many cells were inoculated per tube, so that the fraction of tubes containing transformants of a given kind was higher than in the previous experiment. However,

the results are similar in most respects. It will be noted that both SIII-2 and SIII-N transformants were observed, as the combination of recipient strain and transforming DNA is essentially that of reaction 10 above. According to the hypothesis of unlinked mutations, the SIII-2 class should be rare. The results show that, on the contrary, SIII-2 transformants are probably more frequent than SIII-N transformants, rather than less frequent.

Results of other experiments involving reactions 10 and 11 are given below in Tables 4 and 5, respectively.

Similar results were obtained for four other capsule-deficient mutants (R1, R27, Rz,SIII-1c). They demonstrate that various mutations causing impairment of the ability to synthesize type III capsular polysaccharide are present at separable, recombinable sites of the same transforming agent.

*Mapping the sites of mutation:* Although the mutants R1 and R11 arose independently by mutation from the same encapsulated strain (SIII-2), they are phenotypically alike, and behave similarly in response to the DNA from SIII-N and SIII-2 strains. Nevertheless, they are evidently genotypically dissimilar, since by transforming one of the mutants with the DNA of the other, SIII-2 strains can be reconstituted. Obviously, the mutations affecting capsule synthesis are located at different sites, and as has just been pointed out, these two sites must occur on the same transforming agent (presumably the same DNA molecule).

The possibility arose that the R1 and R11 mutations could be mapped with respect to the SIII-1 and SIII-2 muations, which are also situated on the same transforming agent as those of R1 and R11. In preliminary experiments it became evident that strain R11 in reaction to the DNA of strain SIII-1 produced regularly numerous SIII-N and SIII-2 transformants. Strain R1, on the other hand, in reaction to the same DNA appeared to produce only SIII-2 transformants.

A number of transformation experiments were then undertaken to test whether

TABLE 4

*Short-term exposure of strain Rx to TP SIII-2-str-ery$_2$*

| Transformant class | No. of tubes examined | No. of tubes containing transformants | |
|---|---|---|---|
| ery-resistant | 50 | 4 | |
| str-resistant | 50 | 2 | |
| SIII-2 | 42 | 2 | |
| SIII-N | 42 | 2 | |
| | | Observed | Expected* |
| SIII-2 + SIII-N | 42 | 0 | <1 |
| SIII-2 + str-resist. | 42 | 0 | <1 |
| SIII-2 + ery-resist. | 42 | 0 | <1 |
| SIII-N + str-resist. | 42 | 0 | <1 |
| SIII-N + ery-resist. | 42 | 1 | <1 |
| ery-resist. + str-resist. | 50 | 0 | <1 |

* On basis of independent transfer of transforming agents affecting different functions.
Similar to experiment described in Table 2.
Time of exposure: 20 min. Number of cells transferred from exposed population to each tube: 2600.

TABLE 5

*Short-term exposure of strain R11 to TP SIII-N-str-ery$_2$*

| Transformant class | No. of tubes examined | No. of tubes containing transformants | | No. of transformants per 0.1 ml* |
|---|---|---|---|---|
| ery-resistant | 20 | 18 | | >1000, >1000, >1000, >1000, 802, 781, 752, 683, 632, 534, 531, 507, 492, 282, 249, 163, 147, 119 |
| str-resistant | 20 | 10 | | 718, 502, 422, 417, 356, 257, 152, 52, 33, 2 |
| SIII-N | 20 | 16 | | >100 in each tube |
| SIII-2 | 20 | 5 | | >100 in each tube |
| | | Observed | Expected† | |
| ery-resist. + str-resist. | 20 | 8 | 9.0 | |
| SIII-N + ery-resist. | 20 | 15 | 14.4 | |
| SIII-2 + ery-resist. | 20 | 5 | 4.5 | |
| SIII-N + str-resist. | 20 | 7 | 8.0 | |
| SIII-2 + str-resist. | 20 | 2 | 2.5 | |
| ery-resist. + str-resist. + SIII-N | 20 | 6 | 7.2 | |
| ery-resist. + str-resist. + SIII-2 | 20 | 2 | 2.2 | |
| str-resist. + SIII-N + SIII-2 | 20 | 1 | 2.0 | |
| ery-resist. + SIII-N + SIII-2 | 20 | 4 | 3.6 | |
| ery-resist. + str-resist. + SIII-N + SIII-2 | 20 | 1 | 1.8 | |

* Total no. cells/0.1 ml = $3.2 \times 10^8$.
† On basis of independent transfer of transforming agents affecting different functions.
Similar to experiment described in Table 2.
Time of exposure: 20 min. Number of cells transferred from exposed population to each tube: 3280.

the R1 and R11 mutations differ in their ability to interact with the SIII-1 mutation so as to produce fully encapsulated SIII-N transformants. One group of experiments consisted in exposing R1 and R11 cells to the DNA extracted from streptomycin-resistant SIII-1 strains. The latter included some SIII-1 transformant (SIII-1RT) strains obtained by treatment of encapsulated cells with SIII-1 DNA. Another group of experiments consisted in treating SIII-1 (or SIII-1RT) strains with the DNA extracted from either R1 or R11 mutant strains. The experiments involved 15 to 17 hours of exposure of a growing population of

TABLE 6

*Results of long-term transformation experiments comparing the interaction of R1 or R11 and SIII-1 genomes*

| Exp't no. | Recipient | Donor | str | Frequency of SIII-N | Frequency of SIII-2 |
|---|---|---|---|---|---|
| 369 | R1 | SIII-1-str | c. $10^{-4}$ | $<10^{-4}$ | c. $10^{-3}$ |
| 374 | R1 | SIII-1-str | c. $10^{-5}$ | $<10^{-4}$ | c. $10^{-3}$ |
| | | none | $<10^{-7}$ | $<10^{-4}$ | $<10^{-4}$ |
| 437 | R1 | SIII-1-str | c. $10^{-4}$ | $<10^{-5}$ | c. $10^{-3}$ |
| | | SIII-1 RT no. 21-str | c. $10^{-4}$ | $<10^{-5}$ | c. $10^{-3}$ |
| | | none | $<10^{-7}$ | $<10^{-5}$ | $<10^{-5}$ |
| 507 | R1 | SIII-1 RT no. 19-str | c. $10^{-4}$ | $<10^{-5}$ | c. $10^{-3}$ |
| | | SIII-1 RT no. 31-str | c. $10^{-4}$ | $<10^{-5}$ | c. $10^{-3}$ |
| | | SIII-N-str | $3\times10^{-5}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| | | SIII-N | $<10^{-7}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| 508 | R1 | R36R-str | c. $10^{-4}$ | $<10^{-5}$ | $<10^{-5}$ |
| 447 | R11 | SIII-N | $<10^{-7}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| 510 | R11 | SIII-1-str | c. $10^{-4}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| | | SIII-1 RT no. 19-str | c. $10^{-4}$ | c. $10^{-3}$ | c. $10^{-3}$ |
| | | SIII-N-str | c. $10^{-5}$ | c. $10^{-3}$ | c. $10^{-4}$ |
| | | R36R-str | c. $10^{-4}$ | $<10^{-5}$ | $<10^{-5}$ |
| 521 | R11 | SIII-1 RT no. 31-str | c. $10^{-4}$ | $<10^{-5}$ | c. $10^{-3}$ |
| 576 | SIII-1 | R1-str | c. $10^{-5}$ | $<10^{-5}$ | c. $10^{-3}$ |
| 579 | SIII-1 | R11-str | c. $10^{-4}$ | c. $10^{-3}$ | c. $10^{-4}$ |
| 587 | SIII-1 | R11-str | c. $2\times10^{-5}$ | $1.0\times10^{-3}$ | $1.5\times10^{-4}$ |
| 515 | SIII-1 RT no. 11-str | R1 | ... | $5.5\times10^{-5}$ | c. $10^{-4}$ |
| 519 | SIII-1 RT no. 31-str | R1 | ... | c. $10^{-5}$ | c. $10^{-4}$ |

Summary

| Recipient | Donor | Total no. of experiments | No. of experiments SIII-N:— SIII-2:++ | SIII-N:++ SIII-2:++ | SIII-N:few SIII-2:++ |
|---|---|---|---|---|---|
| R1 | SIII-1 (or SIII-1 RT) | 6 | 6 | 0 | 0 |
| R11 | SIII-1 (or SIII-1 RT) | 3 | 0 | 3 | 0 |
| SIII-1 (or SIII-1 RT) | R1 | 3 | 1 | 0 | 2 |
| SIII-1 | R11 | 2 | 0 | 2 | 0 |

++ = c. $10^{-4}$ or more; — = $<10^{-5}$; few = c. $10^{-5}$

recipient cells to a saturating concentration of the desired DNA (RAVIN 1959). The results of the experiments are tabulated and summarized in Table 6. It will be seen that in a total of five experiments involving an interaction between SIII-1 and R11 genomes, both SIII-N and SIII-2 transformants were always recovered at high frequency. However, in a total of nine experiments involving an interaction between SIII-1 and R1 genomes, SIII-2 transformants were recovered at high frequency in every experiment, but SIII-N transformants were observed in only two of them. Furthermore, in these two experiments, the SIII-N transformants were not as abundant as the SIII-2 type of cell.

These results demonstrate that the SIII-N genome is more difficult to reconstruct when the R1 and SIII-1 genomes are confronted (in a transformation experiment) than when the R11 and SIII-1 genomes are confronted. The locations of the R1, R11 and SIII-1 mutations with respect to each other and to the SIII-2 mutation, could account for this finding, as will be discussed further below.

*Is the R36A mutation a multisite mutation?:* AVERY, MCCARTY and MACLEOD (1944) derived from a type II encapsulated pneumococcus a capsule-deficient mutant of R phenotype which thereafter failed to undergo spontaneous reversions to the encapsulated condition. This strain, R36A, proved suitable, therefore, for transformation studies, since control cultures devoid of transforming DNA never contained encapsulated cells. Many of the early transformation experiments dealing with the capsular character were performed with this strain. In addition, the R36A strain has been carried by serial transfer in a number of different laboratories and has given rise to a large number of subclones and special mutant lines (RAVIN 1959).

As reported previously (RAVIN 1959), the R36A strain which has been carried for the past six years in our laboratory now fails to produce encapsulated transformants when treated with the DNA of encapsulated strains. This failure is not due to any inability of the R36A strain to incorporate genetic determinants other than the one affecting the capsular character. It has been shown (RAVIN 1959) that R36A will be transformed at high frequency to streptomycin resistance, but fails to become encapsulated, when exposed to the DNA extracted from streptomycin-resistant, encapsulated strains.

Since R36A had been transformable for the capsular character in other laboratories, another subclone of the original R36A strain was obtained from DR. FRANCIS SIROTNAK. This strain had been obtained by the latter from DR. ROLLIN HOTCHKISS' laboratory, where it had been derived as a subclone from R36A and designated "R6". We shall refer to it in this paper as R36H in contradistinction to our own subclone, which will be referred to as R36R.

The strain R36H is a competent strain for transformation and has proved to be capable of transforming for a number of genetic characteristics. In particular, it was noted that encapsulated transformants can be produced by treatment of R36H with DNA of an SIII-N strain. It appears, therefore, that the R36H mutation differs from the R36R mutation. As a further test of this conclusion, DNA transforming preparations were made from R36H and R36R after both strains had been previously rendered streptomycin resistant by transfer to them of the

TABLE 7

*Comparison of DNA R36H-str and DNA R36R-str on a variety of host strains*

| Occurrence of | Strain SIII-1 | | Strain Rx | | Strain Rz | | Strain R11 | | Strain R36H | | Strain R36R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DNA R36H-str | DNA R36R-str | DNA R36H-str | DNA R36R-str | DNA R36H-str | DNA R36R-str | DNA R36H-str | DNA R36R-str | DNA R36H-str | DNA R36R-str | DNA R36H-str | DNA R36R-str |
| Streptomycin-resistance transformations | + | + | + | + | + | + | + | + | + | + | + | + |
| Capsular transformations | + | — | + | — | + | — | + | — | — | — | — | — |

+ refers to frequency of transformation exceeding $10^{-5}$.
— refers to frequency of transformation less than $10^{-8}$ in the case of streptomycin-resistance and less than $10^{-5}$ in the case of encapsulation.

*str* marker. These two DNA preparations were then tested on a variety of capsule-deficient strains. The results are recorded in Table 7, and in part of Table 6. It will be seen that encapsulated transformants can be produced by the confrontation of strains SIII-1, Rx, Rz and R11 with the DNA of strain R36H-str. On the contrary, when these same strains were confronted with the DNA of strain R36R-str, no encapsulated transformants were produced. (An exception has been observed only in the case of strain SIII-1 treated with the DNA of TP R36R-str. In two of 16 experiments performed with controls lacking DNA, SIII-N cells were observed at low frequency in the experimental cultures.) Furthermore, no encapsulated transformants were produced when R36H or R36R were exposed to the DNA of either R36R or R36H. It is inferred, therefore, that there is an overlapping of the capsular mutations of R36R and R36H, but that the R36R mutation is more extensive, covering all the capsule-deficient mutations so far examined.

## DISCUSSION

Mutations impairing the ability of Pneumococcus to synthesize capsular polysaccharide are apparently quite common. The experimental results described in this paper lead to the conclusion that the independently arising mutations occur at different sites of the bacterial genome, but that these sites are very closely linked—for the same unit or particle of transforming DNA (presumably a single molecule) carries all of the different sites that mutated to cause capsule deficiency. These results lend further evidence to the conclusion (HOTCHKISS 1956) that the particle of transforming DNA is considerably larger than the genetic factor, the activity of which is being followed in a given experiment. The "capsule mutations" represent the largest series of closely linked genetic sites known in Pneumococcus, for at least nine separable sites (R1, R11, R27, R6H, Rx, Rz, SIII-1, SIII-1c and SIII-2) have been shown to occur on the same transforming particle.

Presumably if more capsule mutations were studied, the number of separable mutable sites would be found to be greater still. Such large clusters of mutable

sites are not unusual in microorganisms, however, for the techniques of transduction and conjugation in bacteria, and of sexual recombination in the molds and yeasts, have uncovered many examples of such cases (for a review, see DEMEREC and HARTMAN 1959).

Particularly relevant to the present study is BENZER's extensive analysis (1957) of a particular region of the genome of bacteriophage T4. It has indicated that a muton, defined as the smallest element in the one-dimensional genetic array which, when altered, results in a mutation, may consist of no more than a very few nucleotides (not more than five). A recon, defined as the smallest element that is interchangeable by genetic recombination, appeared to be smaller than the muton. Taking the molecular weight of DNA to be $1.5 \times 10^7$ (GOODGAL and HERRIOTT 1957), the average molecular weight of a nucleotide to be c. $3.0 \times 10^2$, and assuming the transforming particle to be a molecule of DNA and that this molecule has the double-stranded helical structure of the Watson-Crick model in which a mutation occurs by change in the sequence of nucleotide-pairs in a particular segment of the double helix, it would follow that a few thousand mutations could be brought together by recombination onto a single molecule of DNA.

While this estimate involves a certain degree of error (for example, the muton may be smaller than five nucleotides in length or the molecular weight of DNA may be somewhat less than that assumed above, etc.), it is clear that a very large number of mutations can be represented in a given molecule of DNA. If, in general, a molecule of transforming DNA is concerned with a specific function in the metabolic economy of the cell, it is equivalent to a "complex locus", that is, a region of the genome in which many closely-linked but recombinable mutations may occur to affect a particular cell function. (A "complex locus" may consist of one or more cistrons, a cistron being defined as a region within which any two mutations are functionally noncomplementary when present in the trans position in a cell diploid for this region.)

The importance of the finding of such "complex loci" in an organism, such as Pneumococcus, in which DNA-mediated transformation occurs, is the possibility of conducting physicochemical studies of the DNA molecules which can transfer multiple "pieces of genetic information". What relation exists, for example, between the physical size of a given segment of transforming DNA and the amount of information it can bear? What difference in base composition, if any, is there between the mutable sites of a complex locus? It is to be hoped that the mapping of complex loci in bacteria capable of undergoing DNA-mediated transformations will enable future physicochemical investigations of this sort.

Unfortunately, the linked capsule markers do not lend themselves to quantitative estimates of the recombination distances between markers as readily as do markers that can be screened on selective media. For this reason, linkage is being sought among markers affecting antibiotic resistance or carbohydrate utilization (HOTCHKISS and EVANS 1958; RAVIN and IYER, unpublished). Nevertheless, it has been possible by means of the experiments described in this paper to establish the order of some of the markers in the capsule locus. The order shown in

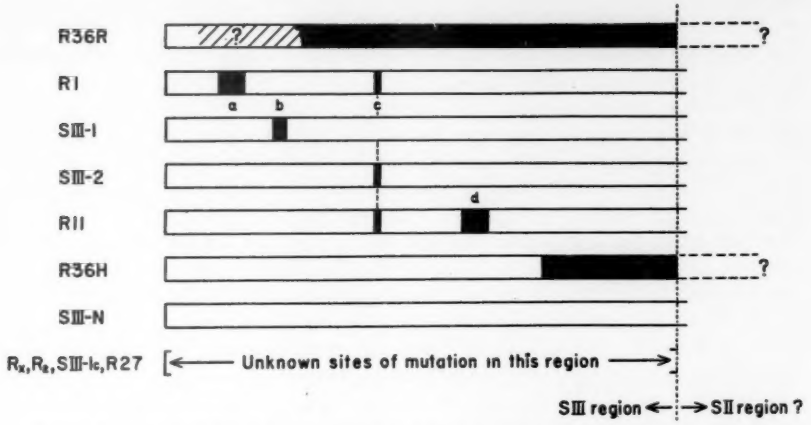


FIGURE 2.—The probable order of several mutations causing impairment of type III polysaccharide synthesis. For explanation, see text.

Figure 2 would account for the results obtained. In this order the SIII-1 and SIII-2 mutations lie between the R1 and R11 mutations, with the SIII-1 mutation lying between the R1 and SIII-2 sites. The rarity of SIII-N transformants in reactions between SIII-1 and R1 would be explained as due to the fact that such transformants could be produced only if two "crossovers" occurred within a short distance, namely, one "crossover" between sites a and b and the other between sites b and c. On the other hand, SIII-N transformants could occur more frequently in reactions between SIII-1 and R11, since a single "crossover" (between b and c) could produce an SIII-N genotype.

It is hypothesized that the original R36A mutation underwent at least one modification in the strain (R36R) maintained in our laboratory. The modification consisted in a mutation or deletion of the major portion of the capsule transforming agent. Thus, interactions are rarely possible between R36R and any of the other capsule-deficient mutations so as to restore the encapsulated condition. The R36R mutation is regarded as including the region mutated in R36H, so that encapsulated transformants cannot be produced from an R36R × R36H interaction; but the R36H mutation is assumed to involve a much smaller segment, so that encapsulated transformants can be produced in interactions between R36H and SIII-1 or R11, for example. The stability of the R36R and R36H mutations can be explained by the extent of the mutation that would have to revert to the wild-type (SIII-N) condition in order for fully encapsulated cells to appear. Since the original R36A mutation occurred in a type II encapsulated pneumoccus, the region of the transforming agent responsible for type II polysaccharide synthesis is assumed to be linked to the type III region, and the R36A mutation is hypothesized to have extended into the type III region (shown to the left of the type II region in Figure 2). This assumption would be compatible with the

findings of AUSTRIAN, BERNHEIMER, SMITH and MILLS (1958), indicating linkage of the regions affecting type I and type III polysaccharide synthesis.

One of the interesting outcomes of these experiments is the confirmation, using an independent test, of a conclusion deduced from studies of transformations in clones derived from single pneumococci suspended in semisolid media (RAVIN 1954). This conclusion was that a transformation requiring a recombination between host genetic material and *part of* the donor transforming DNA (so-called "allogenic" transformations) was due to a unique event and could occur with a greater frequency than transformations due to the substitution of an entire donor DNA agent for a homologous DNA agent in the host cell (so-called "autogenic" transformations). This finding limits the number of models that can be proposed for the mechanism of transformation. A model that is eliminated, for example, is that replication of the transforming DNA agent precedes integration into the bacterial genome. If replication did occur, either the replica or the parental transforming particle would have to disappear, since autogenic and allogenic transformants do not both descend from a recipient cell infected by only a single transforming agent. An acceptable hypothesis is that all or some part of the transforming particle is integrated into the nascent recipient genome as the latter is being completed during replication of bacterial DNA. When the integration occurs, whatever portion of the original transforming agent is unused is discarded. The actual integration might also occur by a copy-choice mechanism, presumably if the transforming DNA synapses in a precise way with its homologue in the existing bacterial genome and the nascent genome is produced by copying alternately from the exogenous transforming agent and the endogenous homologue; in this case, after the copying takes place the template is destroyed.

Certain of the results described in this paper suggest that the transforming particle may be transmitted in a unilinear fashion for an appreciable number of generations before the actual process of genetic integration occurs. This view is suggested by the distribution of the number of transformants in parallel tubes inoculated with small samples of cells treated with transforming DNA. The highest frequency of transformants observed in a given tube is what would be expected if: (1) the transforming DNA were incorporated into one member of a still-attached pair of cocci (or into one nucleus of a binucleate cell); and (2) integration occurred within the first generation; but (3) one cell division had to occur before the cell (or nucleus) containing the transforming DNA were separated from its sister not containing a transforming agent. However, a considerable number of tubes contained a far lower frequency of transformants. In some tubes there was a hundredfold decrease in the proportion of transformed to untransformed cells compared with the proportion of cells containing transforming DNA actually inoculated into those tubes. To account for this result on the grounds that each "cell" in a pneumococcal culture is actually a single colony-forming chain of cells, would require the additional assumption that an appreciable number of the colony-forming units in a pneumococcal culture consisted of a hundred cocci. Direct microscopic counts of the chain-lengths in an

exponentially growing pneumococcal culture, such as the ones used in these experiments, do not corroborate this assumption. Actually it is found that over 60 percent of the colony-forming units are at most diplococci (chains of two cells), and that under ten percent contain more than eight cocci in a chain. No chains of more than 20–30 cocci were ever observed in this class, however.

An alternative explanation for the low frequencies of transformants observed in certain tubes is that the transformants have a slower growth rate than the untransformed cells. This explanation faces a number of objections. First of all, why should the growth rate of the transformant differ from the normal growth rate in a nonuniform way among the parallel tubes—in some tubes varying unappreciably, in others considerably? Secondly, a distribution of the number of transformants is found regardless of the marker involved, such as *ery*$_2$ or *str*. Why should a similar impairment of growth rate be found for all types of transformed cells? Finally, reconstruction experiments in which transformed cells (*str* or *ery*$_2$) are mixed with untransformed cells do not indicate any difference in growth rate.

One possibility remains, however: that those cells which have just absorbed transforming DNA are set back, to a variable extent, in their growth. While other investigations have not borne out this possibility (HOTCHKISS 1957), it must be determined whether the particular conditions employed in the present experiments bring about this effect. Indeed, other studies of integration lag (HOTCHKISS 1956; EPHRUSSI-TAYLOR 1959) have not revealed such long lags as must be assumed to occur to account for the results of the present experiments. A definitive conclusion as to the cause of the distribution of the number of transformants in parallel cultures cannot, therefore, be made at this time.

## SUMMARY

Several mutations resulting in impaired synthesis of type III capsular polysaccharide have been shown to occur at different sites on the same unit or particle of transforming DNA. Furthermore, the sites of some of these mutations relative to each other in a linear series could be determined. Certain mutations behaved in recombination as though they extended over long segments of the transforming DNA, covering several separable mutable sites.

The conclusion is confirmed, by an independent test, that a transformation reaction requiring a recombination of part of the donor transforming DNA with host genetic material occurs as the result of a unique event. It is consistent with available evidence that all transformations involve recombinations with some part of the transforming DNA particle.

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# BLOOD GROUPS IN SHEEP. II. THE B SYSTEM[1]

BENJAMIN A. RASMUSEN[2]

*The School of Veterinary Medicine,*
*University of California, Davis, California*

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EXAMINATION of the cross-reactions of numerous bovine and ovine isoimmune antisera with red cells of cattle and sheep, reported by STORMONT, SUZUKI and RASMUSEN (1957), has provided serological evidence for a system of blood groups in sheep homologous to the complex B system of bovine blood groups described by STORMONT, OWEN and IRWIN (1951). This report presents further evidence for the B system of sheep. It is primarily concerned with the genetic elucidation of B phenogroups in sheep and a description of their cross-reactions with diagnostic reagents prepared from isoimmune and heteroimmune antisera.

## MATERIALS AND METHODS

Bovine and ovine isoimmune antisera and heteroimmune antisera produced in sheep against red cells of cattle were provided by DR. CLYDE STORMONT for these studies. The procedure followed in the production of bovine isoimmune antisera is described by STORMONT (1950) and of ovine isoimmune antisera by RASMUSEN, STORMONT and SUZUKI (1960). The heteroimmune antisera were produced by immunizing sheep with pooled erythrocytes from three cows, XX, 88 and 2758. Their phenogroup formulas in the B system were known to be, respectively, $IE'_1/O_xE'_38$, $BGKO_2Y_1A'E'_3K'4,6,8,/BGKO_2Y_1A'E'_3K'4,6,8$ and $BGKE'_27,8/O_xD'E'_38$. Eighteen sheep were immunized by two 30-ml injections made three days apart, and antisera were collected nine days after the last injection. While some of these heteroimmune antisera contained antibodies which cross reacted with the red cells of individual sheep, the cross-reactions were generally too weak to be of use in blood typing of sheep. Consequently, approximately four months later the sheep were given an additional injection of 30 ml of red cells from the same donor cows, and antisera were collected nine days later. Seven of these antisera proved to be of use as a source of blood-typing reagents for the present study.

Absorptions with erythrocytes of individual sheep were performed on each heteroimmune and isoimmune antiserum that contained antibodies reactive with the red cells of sheep in order to separate any of the populations of antibodies

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[2] Present address: Department of Animal Science, University of Illinois, Urbana.

which might be present in them. In some cases, trial absorptions showed that an antiserum contained but a single population of antibodies so that no absorptions were necessary for the preparation of a reagent. The antisera were then titrated to determine an appropriate dilution for testing. The titrations were set up as a series of doubling dilutions, and the greatest dilution which resulted in complete or nearly complete lysis with most of the reactive cells at the end of 2½ to 3 hours was chosen as the dilution for testing. The dilution chosen was usually one fourth. Frequently, natural anti-R was present in antisera from group O sheep. These interfering, natural antibodies were routinely removed by two successive absorptions (15 minutes and 30 minutes) of the undiluted serum with washed, human erythrocytes of group A, using a ratio of cells to serum of 1:4 per absorption.

When an antiserum was found by trial absorptions to contain mixed populations of antibodies or subfractions of what appeared to be a single population, the tests of the individual absorptions were compared, and appropriate cells were selected in order to prepare reagents containing but a single population or fraction of antibodies. In many cases, trial absorptions were performed with a combination of absorbing bloods to produce such reagents.

In most cases, it was possible to exhaust all of the antibodies for the absorbing bloods by means of two successive absorptions (15 minutes and 30 minutes) when a ratio per absorption of 1:2 of cells to diluted serum was used. After trial tests and titrations, appropriate dilutions of the reagents were selected for use in the blood-typing tests. Usually the antiserum was diluted one half for absorption and one half for testing, making a final dilution of one fourth of the original antiserum.

Reagents were named by letter for convenience. Those which closely paralleled any previously prepared reagent in reactivity were designated by the same letter as the original reagent.

The procedure followed in the blood-typing tests was described by RASMUSEN (1958). Briefly, two drops (0.10 ml) of diluted antiserum and one drop (0.05 ml) of a two to three percent suspension of washed erythrocytes were mixed in a 10 × 75 mm glass tube. After a few minutes one drop (0.05 ml) of complement was added, the tubes were again shaken, and the test was read at approximately one half, 2½ and 4½ hours later. The tubes were shaken after each of the first two readings. Rabbit complement, absorbed at 4°C to remove natural heterohemolysins for sheep, was found to be satisfactory for use with all of the reagents reacting in the B system.

## EXPERIMENTAL RESULTS

*Family studies:* Materials for the genetic studies consisted of blood samples from 214 ewes, their 255 lambs and the 15 rams which sired the lambs. These were largely the same blood samples utilized in the genetic studies of the X–Z system (RASMUSEN 1958).

Although each of the blood samples was tested with 44 reagents in addition to the six reagents utilized in the tests for R–O and X–Z phenogroups, this discussion

is confined to the reactions obtained with those reagents shown to be reactive with phenogroups in the B system. These were the 12 reagents named B′, E, $E_x$, E′, E′ + I′, $I_2$, N, $P_1$, $P_x$, Q, S and $U_2$ prepared from ovine isoimmune antisera, the nine reagents B, B′, $I_x$, I′, $O'_1$, $O'_x$, $P_2$, S and Y prepared from bovine isoimmune antisera and the seven reagents E, $I_1$, $T_1$, $T_2$, $T_3$, $U_1$ and $U_3$ prepared from ovine heteroimmune antisera against cattle blood. Reagents with the same name which are designated with subscripts bear subtyping relationships to each other similar to those described by STORMONT (1950) in studies of bovine isoimmune antisera. In these cases, the more broadly cross-reactive reagent was designated by a subscript "x", or "3" or "2", so that the order from the least cross-reactive to the most cross-reactive reagent in such sets would be 1, 2, 3 . . . x. In two cases, two reagents were found to bear a linear subtyping relationship to a more widely cross-reactive reagent, but were not themselves linearly related. The reactions of reagents $O'_1$ and Q were always within those of $O'_x$. Similarly, reactions of $I_2$ and I′ were within those of $I_x$, as were the reactions of $I_1$ which was simply a subtype of $I_2$.

The red cells of Shropshire ram 114 were lysed by reagents B, B′, E, $E_x$, $O'_1$, $O'_x$, Q, $T_3$, $U_1$, $U_2$ and $U_3$. When the erythrocytes of any of the 51 lambs of ram 114 were lysed by any of these nine reagents which did not lyse the erythrocytes of the dams of these lambs, the reagents lysing the blood of the lamb fell into one of the two groups: B, B′, E, $E_x$, $O'_1$, $O'_x$, $T_3$, $U_1$, $U_2$, and $U_3$, as opposed to $O'_x$ and Q. None of the reactions of the red cells of the lambs of ram 114 fell outside these two groups.

On the basis of cross-reactions with the B-system reagents, the two B phenogroups of ram 114 may be written $BB'EO'_1T_3U_1$ and $O'_xQ$. Twenty-six of his lambs inherited $BB'EO'_1T_3U_1$ from him, 21 inherited $O'_xQ$, and four ewes and their lambs had the same B phenogroups as the ram so that his contribution to these lambs could not be determined.

Table 1 shows the B phenogroups contributed by ram 114 to 20 of his lambs: ten to which he contributed $BB'EO'_1T_3U_1$, and ten to which he contributed $O'_xQ$. The probable phenogroups of the dams of these lambs are also listed. The lambs were selected to show the various combinations of phenogroups observed in blood samples from this sire family, as well as the inheritance of the phenogroups.

Table 2 shows the inheritance of the two B phenogroups in 20 selected progeny of a Targhee ram, 9501T, whose cells were lysed by reagents B, B′, E, $E_x$, $I_1$, $I_2$, $I_x$, I′, $P_1$, $P_2$, S, $T_1$, $T_2$, $T_3$ and Y. Eighteen of his lambs inherited phenogroup $EE'I_xI'P_1T_1$ from him and 12 inherited $BB'E_xI_1SY$. Thus the reagents E′, $I_1$, $I_2$, $I_x$, I′, $P_1$, $P_2$, $P_x$, S, $T_1$, $T_2$ and Y, in addition to B, B′, E, $E_x$, N, $O'_1$, $O'_x$, Q, $T_3$, $U_1$, $U_2$ and $U_3$ in the family of ram 114 were clearly shown to react in the B system. The families of rams 114 and 9501T were selected for illustration since the B phenogroups of the two rams reacted with all the B reagents of the present study with but one exception, reagent N. The N reagent was shown to be reacting in the B system in the family of ram 114 (Table 1) as well as in the families of rams 5020T, 53035T, 2435XW and 396XW (Table 3).

All of the sire families were studied in a manner similar to the studies of the

TABLE 1

*Inheritance of B phenogroups of ram 114 (Shropshire) by 20 of his offspring*

| Mating number | Dam's number | Probable phenogroups of dam | | Offspring's number | Phenogroups of offspring from sire | from dam |
|---|---|---|---|---|---|---|
| 1 | 739 | $O'_xQ$ | /BNY | 401 | $O'_xQ$ | /$O'_xQ$ |
| 2 | 731 | $O'_xQ$ | /BB'EO'$_1$T$_3$U$_1$ | 417 | $O'_xQ$ | /$O'_xQ$ |
| 3 | 227 | EO'$_1$Y | /BNY | 423 | $O'_xQ$ | /EO'$_1$Y |
| 4 | 181 | BNY | /$O'_xQ$ | 409 | $O'_xQ$ | /BNY |
| 5 | 23 | BNY | /BEE'P$_2$T$_1$U$_1$Y | 436 | $O'_xQ$ | /BNY |
| 6 | 21 | BEE'P$_2$T$_1$U$_1$Y | /BNY | 477 | $O'_xQ$ | /BEE'P$_2$T$_1$U$_1$Y |
| 7 | 231 | BB'EO'$_1$U$_3$ | /BB'EO'$_1$T$_3$U$_1$ | 420 | $O'_xQ$ | /BB'EO'$_1$U$_3$ |
| 8 | 101 | BB'EO'$_1$T$_3$U$_1$ | /BEE'P$_2$T$_1$U$_1$Y | 391 | $O'_xQ$ | /BB'EO'$_1$T$_3$U$_1$ |
| 9 | 167 | NY | /BNY | 421 | $O'_xQ$ | /NY |
| 10 | 578 | NY | /BEE'P$_2$T$_1$U$_1$Y | 482 | $O'_xQ$ | /NY |
| 10 | 578 | BEE'P$_2$T$_1$U$_1$Y | /NY | 481 | BB'EO'$_1$T$_3$U$_1$ | /BEE'P$_2$T$_1$U$_1$Y |
| 11 | 743 | BEE'P$_2$T$_1$U$_1$Y | /BB'EO'$_1$T$_3$U$_1$ | 455 | BB'EO'$_1$T$_3$U$_1$ | /BEE'P$_2$T$_1$U$_1$Y |
| 12 | 222 | BB'EO'$_1$T$_3$U$_1$ | /$O'_xQ$ | 444 | BB'EO'$_1$T$_3$U$_1$ | /BB'EO'$_1$T$_3$U$_1$ |
| 13 | 111 | E'P$_2$T$_2$U$_3$Y | /BNY | 474 | BB'EO'$_1$T$_3$U$_1$ | /E'P$_2$T$_2$U$_3$Y |
| 14 | 108 | $O'_xQ$ | /BNY | 392 | BB'EO'$_1$T$_3$U$_1$ | /$O'_xQ$ |
| 15 | 182 | EO'$_1$Y | /BNY | 422 | BB'EO'$_1$T$_3$U$_1$ | /EO'$_1$Y |
| 16 | 662 | EO'$_1$Y | /NY | 476 | BB'EO'$_1$T$_3$U$_1$ | /EO'$_1$Y |
| 17 | 206 | BNY | /BB'EO'$_1$T$_3$U$_1$ | 473 | BB'EO'$_1$T$_3$U$_1$ | /BNY |
| 18 | 826 | BNY | /BNY | 479 | BB'EO'$_1$T$_3$U$_1$ | /BNY |
| 19 | 96 | $O'_xQY$ | /BNY | 475 | BB'EO'$_1$T$_3$U$_1$ | /$O'_xQY$ |

TABLE 2

*Inheritance of B phenogroups of ram 9501T (Targhee) by 20 of his offspring*

| Mating number | Dam's number | Probable phenogroups of dam | | Offspring's number | Phenogroups of offspring from sire | from dam |
|---|---|---|---|---|---|---|
| 1 | 7477T | NY | /BEO'$_x$QY | 1589GT | EE'I$_x$I'P$_1$T$_1$ | /NY |
| 2 | 7287T | NY | /BB'O'$_1$Q | 1678GT | EE'I$_x$I'P$_1$T$_1$ | /NY |
| 3 | 8946T | BB'EO'$_x$T$_3$U$_2$ | /EE'I$_x$I'P$_1$T$_1$ | 1658GT | EE'I$_x$I'P$_1$T$_1$ | /BB'EO'$_x$T$_3$U$_2$ |
| 4 | 8904T | EE'I$_x$I'P$_1$T$_1$ | /BB'E$_x$I$_1$S | 1877GT | EE'I$_x$I'P$_1$T$_1$ | /EE'I$_x$I'P$_1$T$_1$ |
| 5 | 9910T | BB'O'$_x$ | /BB'E$_x$I$_1$SY | 2068GT | EE'I$_x$I'P$_1$T$_1$ | /BB'O'$_x$ |
| 6 | 7817T | BB'O'$_x$ | /NY | 2107GT | EE'I$_x$I'P$_1$T$_1$ | /BB'O'$_x$ |
| 7 | 7641T | BB'E$_x$I$_1$S | /NY | 2122GT | EE'I$_x$I'P$_1$T$_1$ | /BB'E$_x$I$_1$S |
| 8 | 9387T | BB'E$_x$I$_1$S | /EE'I$_x$I'P$_1$T$_1$ | 1641GT | EE'I$_x$I'P$_1$T$_1$ | /BB'E$_x$I$_1$S |
| 9 | 127GT | BB'E$_x$I$_1$SY | /NY | 1461GT | EE'I$_x$I'P$_1$T$_1$ | /BB'E$_x$I$_1$SY |
| 10 | 9116T | BB'E$_x$I$_1$SY | /BEO'$_x$QY | 2241GT | EE'I$_x$I'P$_1$T$_1$ | /BB'E$_x$I$_1$SY |
| 10 | 9116T | BB'E$_x$I$_1$SY | /BEO'$_x$QY | 2240GT | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$SY |
| 11 | 7941T | BB'E$_x$I$_1$SY | /NY | 1594GT | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$SY |
| 12 | 8864T | BB'E$_x$I$_1$SY | /BB'EO'$_x$T$_3$U$_2$ | 2261GT | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$SY |
| 13 | 6246T | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$SY | 2179GT | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$SY |
| 14 | 6517T | BB'E$_x$I$_1$S | /BB'O'$_1$Q | 1687GT | BB'E$_x$I$_1$SY | /BB'E$_x$I$_1$S |
| 15 | 8659T | BB'O'$_x$ | /EE'I$_x$I'P$_1$T$_1$ | 2043GT | BB'E$_x$I$_1$SY | /BB'O'$_x$ |
| 16 | 9475T | BB'EO'$_x$T$_3$U$_2$ | /BB'E$_x$I$_1$SY | 1607GT | BB'E$_x$I$_1$SY | /BB'EO'$_x$T$_3$U$_2$ |
| 17 | 8809T | BY | /EE'I$_x$I'P$_1$T$_1$ | 1431GT | BB'E$_x$I$_1$SY | /BY |
| 18 | 9570T | EE'I$_x$I'P$_1$T$_1$ | /BB'ENO'$_x$Q | 1783GT | BB'E$_x$I$_1$SY | /EE'I$_x$I'P$_1$T$_1$ |
| 19 | 8687T | EE'I$_x$I'P$_1$T$_1$ | /BEO'$_x$QY | 2007GT | BB'E$_x$I$_1$SY | /EE'I$_x$I'P$_1$T$_1$ |

TABLE 3

*B phenogroups of 15 rams*

| Breed | Ram no. | Phenogroup formula | Number of lambs inheriting each phenogroup |
|---|---|---|---|
| Shropshire | 114 | $BB'EO'_1T_3U_1/O'_xQ$ | 26/21 |
| Shropshire | 30 | $BB'EO'_1T_3U_1/EO'_1Y$ | 24/24 |
| Suffolk | 669-0 | $O'_xQ$ $/O'_1$ | 7/1 |
| Suffolk | 76650 | $O'_xQ$ $/O'_1$ | 3/1 |
| Suffolk | 105334 | $BB'E_xI_1Y$ $/BO'_xY$ | 7/5 |
| Grade Suffolk | 21 | B $/E'P_2ST_1$ | 9/12 |
| Corriedale | 412 | $BO'_1Y$ $/BEO'_xQY$ | 11/11 |
| Targhee | 5020T | $BB'ENO'_xQ$ $/BB'E_xI_1Y$ | 10/3 |
| Targhee | 53035T | $BB'ENO'_xQ$ /BEY | 8/8 |
| Targhee | 9501T | $BB'E_xI_1SY$ $/EE'I_xI'P_1T_1$ | 12/18 |
| Rambouillet | 458RW | $BB'E_xO'_xQS$ $/BB'E_xO'_xQS$ | 10 |
| Rambouillet | 2516XW | $BB'E_xO'_xQS$ $/B'O'_1Y$ | 2/1 |
| Rambouillet | 4375RW | $BB'EO'_1P_1Q$ $/B'O'_xS$ | 6/8 |
| Rambouillet | 2435XW | $E'NP_3T_2Y$ $/E'NP_3T_2Y$ | 9 |
| Rambouillet | 396XW | $BB'E_xI_1S$ $/E'NP_3T_2Y$ | 3/2 |

families of Shropshire ram 114 and Targhee ram 9501T. In each family an attempt was made to postulate as few phenogroups as possible. Nevertheless, postulation of a total of 52 different B phenogroups was required to account for the cross-reactions observed in comparing the reactions of the red cells of the lambs and of their parents. This number is a minimum for the flocks studied; more phenogroups might have been diagnosed if more reagents had been available and if the matings had been suitable for revealing all of the phenogroups in each flock. Table 4 lists the cross-reactions of the 52 B phenogroups which were postulated. Code designations, which will be discussed elsewhere in this report, are also given in Table 4.

*Cross-reactions of the B phenogroups:* Shropshire ram 30 was used in the same flock as ram 114. He contributed $BB'EO'_1T_3U_1$, already identified in ram 114, to ten of his lambs, and $EO'_1Y$ to 24 lambs (Table 3). The three phenogroups $BB'EO'_1T_3U_1$, $O'_xQ$ and $EO'_1Y$, together with a fourth, BNY, comprised over 80 percent of the phenogroups in the flock in which these two rams were used. Phenogroup BNY was deduced from serological evidence as well as from a study of phenogroups inherited by the lambs from their dams. Bloods from this flock which were lysed by reagent N were invariably lysed by reagent Y, known to be reacting in the B system, as evidenced by phenogroup $EO'_1Y$ which appeared as the genetic alternative to $BB'EO'_1T_3U_1$ in the progeny of ram 30. Most of the bloods lysed by reagents N and Y were also lysed by reagent B (the only exceptions were those whose two B phenogroups were NY plus any other phenogroup which did not result in lysis by the B reagent). Phenogroup BNY could be frequently identified, and was most easily seen in bloods of the formula $BNY/O'_xQ$.

The B phenogroups were characterized not only by the reagents with which they reacted, but also by the intensity or degree of cross-reactions of the reagents.

TABLE 4

*Cross-reactions of phenogroups of the B system of sheep*

| Code no. | 1. The E′ set | Code no. | 2. The I set | Code no. | 3. The Q set |
|---|---|---|---|---|---|
| E′ 1 | $BEE'P_2T_1U_1Y$ | I 1 | $BB'E_xI_1N$ | Q 1 | $BB'ENO'_xQ$ |
| E′ 2 | $BEE'P_2T_2U_2$ | I 2 | $BB'E_xI_1NS$ | Q 2 | $BB'EO'_1P_1Q$ |
| E′ 3 | $EE'I_xI'P_1T_1$ | I 3 | $BB'E_xI_1S$ | Q 3 | $BB'EO'_xQST_2U_2$ |
| E′ 4 | $E'NP_3T_2Y$ | I 4 | $BB'E_xI_1SY$ | Q 4 | $BB'E_xO'_xQS$ |
| E′ 5 | $E'P_2ST_1$ | I 5 | $BB'E_xI_1Y$ | Q 5 | $BB'O'_1Q$ |
| E′ 6 | $E'P_2T_2U_3Y$ | I 6 | $BEI_xI'ST_2U_1$ | Q 6 | $BB'O'_xQSY$ |
| | | I 7 | $BI_xY$ | Q 7 | $BEO'_xQY$ |
| | | I 8 | $I_1Y$ | Q 8 | $B'O'_xQ$ |
| | | I 9 | $I_xY$ | Q 9 | $O'_xQ$ |
| | | | | Q 10 | $O'_xQY$ |

| Code no. | 4. The O′ set | Code no. | 4. The O′ set | Code no. | 5. The B set (residual) |
|---|---|---|---|---|---|
| O′ 1 | $BB'EO'_1SY$ | O′ 10 | $BO'_1Y$ | B 1 | — (no cross-reactions) |
| O′ 2 | $BB'EO'_1T_3U_1$ | O′ 11 | $BO'_xY$ | | |
| O′ 3 | $BB'EO'_1U_3$ | O′ 12 | $B'EO'U_3Y$ | B 2 | B |
| O′ 4 | $BB'EO'_xT_3U_2$ | O′ 13 | $B'NO'_1$ | B 3 | BE |
| O′ 5 | $BB'O'_1$ | O′ 14 | $B'O'_1$ | B 4 | BENY |
| O′ 6 | $BB'O'_x$ | O′ 15 | $B'O'_1Y$ | B 5 | BEY |
| O′ 7 | $BENO'_1$ | O′ 16 | $B'O'_xS$ | B 6 | BNY |
| O′ 8 | $BEO'_1T_2U_2$ | O′ 17 | $EO'_1Y$ | B 7 | BY |
| O′ 9 | $BO'_1$ | O′ 18 | $O'_1$ | B 8 | NY |
| | | O′ 19 | $O'_1S$ | | |

Table 5 summarizes the tests of the bloods in which phenogroups $BB'EO'_1T_3U_1$, $EO'_1Y$, $O'_xQ$ and BNY were present in various combinations in this flock. Degrees of hemolysis are listed as 0, 1, 2, 3 and 4 (complete hemolysis). Although some variability in degrees of hemolysis of bloods of the same phenogroup formula was observed, it was never marked. This table summarizes the modal degrees of hemolysis for bloods of each formula with pertinent reagents. It shows only the degree of hemolysis seen five hours after the tests were set up and, therefore, does not take into account the rate of hemolysis with individual reagents. Nevertheless, even here differences are apparent; the $O'_x$ reagent, for example, lysed cells of three of the four phenogroups, but to differing degrees. Bloods with phenogroup $O'_xQ$ were characterized by rapid, complete hemolysis with the $O'_x$ reagent, those with phenogroup $EO'_1Y$ by moderate hemolysis, and those with $BB'EO'_1T_3U_1$ by very slow, slight hemolysis. Differences between homozygotes and heterozygotes in rates and degrees of reaction with many of the reagents was observed throughout this study. Such dosage effects were also observed in some cases between certain phenogroups in heterozygous combinations (especially when the reagent showing dosage reacted with each of the two phenogroups so combined) but also occasionally when this was not the case. For example, dosage was observed with the $O'_1$, $O'_x$, $U_1$ and $U_2$ reagents so that bloods of the phenogroup formula $BB'EO'_1T_3U_1/BB'EO'_1T_3U_1$ were lysed more completely by each of these four reagents than were bloods of formula $BB'EO'_1T_3U_1/BNY$, as

TABLE 5

*Summary of the cross reactions of four B phenogroups of Shropshire sheep*

| Phenogroup formulas | | No. of bloods | Reagents and antiserum numbers* B C 31 | B′ C 71 | B′ S 4 | E S 63 | E SC 48 | $E_x$ S 34 | N S 37 | $O'_1$ C 71 | $O'_x$ C 65 | Q S 6 | $T_3$ SC 45 | $U_1$ SC 53 | $U_2$ S 25 | $U_3$ SC 54 | Y C 122 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $O'_xQ$ | $/O'_xQ$ | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| $O'_xQ$ | $/BB'EO'_1T_3U_1$ | 23 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 3 | 4 | 4 | 3 | 1 | 3 | 4 | 0 |
| $O'_xQ$ | $/EO'_1Y$ | 5 | 0 | 1 | 0 | 3 | 3 | 3 | 0 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 4 |
| $O'_xQ$ | /BNY | 17 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 4 |
| $BB'EO'_1T_3U_1$ | $/BB'EO'_1T_3U_1$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 4 | 2 | 0 | 3 | 3 | 4 | 4 | 0 |
| $BB'EO'_1T_3U_1$ | $/EO'_1Y$ | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 4 | 3 | 0 | 3 | 1 | 3 | 4 | 4 |
| $BB'EO'_1T_3U_1$ | /BNY | 13 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 1 | 0 | 2 | 1 | 3 | 4 | 4 |
| $EO'_1Y$ | $/EO'_1Y$ | 1 | 0 | 1 | 0 | 3 | 3 | 3 | 0 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 4 |
| $EO'_1Y$ | /BNY | 20 | 4 | 1 | 0 | 3 | 3 | 3 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 4 |
| BNY | /BNY | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |

* Bovine isoimmune antisera are coded C, ovine isoimmune antisera are coded S and ovine heteroimmune antisera against cattle red cells are coded SC. Bloods of the phenogroup formulas listed in this table did not react with reagents E′ (S21), I′ + E′ (S56), $I_1$ (SC38), $I_2$ (S63), $I_x$ (C31), I′ (C10), $P_1$ (S18), $P_2$ (C47), $P_x$ (S26), S (S35), S (C27), $T_1$ (SC55) and $T_2$ (SC44).

may be seen in Table 5. Similarly, cells of formula $O'_xQ/BB'EO'_1T_3U_1$ were lysed more completely by the $O'_1$ reagent than were those of formula BNY/$BB'EO'_1T_3U_1$. Observations of this kind were very helpful in diagnosing phenogroups, as the reagents were quite consistent in their reactions with bloods of each phenogroup formula.

Phenogroup $EO'_1Y$ was characterized in part by partial hemolysis with the B′ reagent prepared from a bovine isoimmune antiserum, C71. However, absorption of this reagent with bloods of formula $EO'_1Y/O'_xQ$ or $EO'_1Y$/BNY removed this weakly cross-reactive fraction without removing the principal fraction which paralleled the reactions of the B′ reagent prepared from an ovine isoimmune antiserum, S4. Minor differences in reagents of this order often proved helpful in the diagnosis of the phenogroups. Not all such information was incorporated into the phenogroup formulas.

The results of the tests of the Targhees showed numerous examples of graded cross reactions, just as did those of the Shropshires. For example, the phenogroups listed as $BB'E_xI_1S$ and $BB'E_xI_1SY$ in Tables 2, 3 and 4 appear to be very similar on the basis of a comparison of their phenogroup formulas, but cells with the phenogroup $BB'E_xI_1S$ were lysed much more slowly by the B reagent, more rapidly by the $E_x$ reagent and more slowly by the $I_1$ and $I_2$ reagents than those with $BB'E_xI_1SY$, so that in addition to the reaction with the Y reagent the two phenogroups were clearly distinguishable.

The effects of dosage were observed in many cases in addition to those listed in Table 5. Comparison of the cross-reactions of the reagents with bloods with a specific phenogroup in one dose and in two doses revealed differences in reactivity in most cases in which the reagent did not completely lyse the erythrocytes of the heterozygote. Ram 4458RW was homozygous for $BB'E_xO'_xQS$, and ram

2516XW was heterozygous for this phenogroup; dosage was observed with the B and Q reagents, and the E reagent prepared from a heteroimmune antiserum (SC48) slightly lysed the cells of the homozygous ram. Ram 2435XW was homozygous for phenogroup E′NP$_3$T$_2$Y, and ram 396XW was heterozygous for this phenogroup. Dosage was observed with one E′ reagent and with the N reagent. Reagents E (SC48) and I$_1$ also lysed the erythrocytes of the homozygote, apparently due to residual antibodies in these reagents which were comparable to those of the E′ reagent, and which were of sufficient concentration to lyse erythrocytes of the homozygote E′NP$_3$T$_2$Y but not those of heterozygotes.

As a general rule, reagents prepared from bovine isoimmune antisera produced more rapid and complete hemolysis, or more uniformly lysed reactive cells than many of those of ovine origin, so that cells lysed by these reagents were usually very clearly distinguished from those not lysed. Nevertheless, they were actually less useful in identifying individual phenogroups on the basis of degrees of cross-reactions than were the reagents which produced different degrees of hemolysis with reactive bloods of different phenogroup formulas.

In cases where the reactions were of a doubtful nature (e.g., very slow or very weak hemolysis) absorption tests were performed, when sufficient quantities of blood were available, to determine whether the antibodies could be absorbed from the reagent by erythrocytes with the phenogroup in question. If the antibodies were completely absorbed, the reagent name was included in the formula of cross reactions for that phenogroup. Absorptions sometimes failed to give clear-cut results, so that in some cases antibodies might be partially absorbed, and in others, completely so. Therefore, designating cross-reactions in the phenogroup formulas was occasionally somewhat arbitrary.

*Coding the phenogroups:* In view of the fact that reagents for B-system phenogroups of sheep differed widely in the cross reactions they exhibited, and the possibility that another set of reagents could be developed which for the most part might be quite unlike those used in this study, it was considered advisable to code the 52 B phenogroups in order to avoid undue emphasis on the battery of reagents used to diagnose them. The coded phenogroups (Table 4) have been arranged in sets based on the reactions with certain key reagents which are likely to be reproduced in future studies. These sets are similar in arrangement to those proposed by STORMONT (1955) for B phenogroups of cattle.

(1) The E′ set of B phenogroups (Table 4) includes all phenogroups which reacted to some degree with E′ reagents. (2) The I set (Table 4) includes all phenogroups which reacted with the I$_x$ reagent excepting those included in the E′ set. Of the phenogroups observed, only one in the E′ set, (coded E′ 3) cross-reacted with the I$_x$ reagent. (3) The Q set (Table 4) includes the phenogroups which reacted with the Q reagent. (4) The O′ set (Table 4) includes the phenogroups which reacted with the O′$_1$ and/or the O′$_x$ reagents, excepting those already assigned to the Q set. (5) The B set (Table 4) is the residual set and includes all phenogroups not in the foregoing sets.

The "key" E′ reagents were prepared from ovine isoimmune antisera, coded S21 and S56, which have also been used in this laboratory as sources of I and E′$_3$

reagents for cattle blood-typing tests. Three of the other four key reagents ($I_x$, $O'_1$ and $O'_x$) were prepared from bovine isoimmune antisera, coded C31, C71 and C65, which have served, respectively, as sources of the cattle reagents named I, $O_1$ and $O_x$. Thus, four of five key reagents in the sheep tests were from antisera which have served as important sources of B-system reagents in the cattle tests.

Table 6 lists the breeds of sheep studied and the phenogroups found in each breed. As can be seen from the table, some phenogroups were found in more than one breed. The asterisks which designate the phenogroups which were relatively

TABLE 6

*Distribution by breed of B phenogroups of sheep*

| Rambouillet | Targhee | Corriedale | Shropshire | Suffolk |
|---|---|---|---|---|
| E′ 4* | E′ 3* | I 3 | E′ 1 | E′ 1 |
| I 1 | I 3* | Q 7 | E′ 6 | I 5 |
| I 2 | I 4* | Q 8 | Q 9* | I 7 |
| I 3* | I 5 | O′ 10 | Q 10 | I 8 |
| I 4 | Q 1 | O′ 11 | O′ 2* | I 9 |
| I 5 | Q 5 | O′ 13 | O′ 3 | Q 8 |
| Q 1* | Q 7 | B 1 | O′ 12 | Q 9* |
| Q 2* | O′ 4 | | O′ 17* | Q 10 |
| Q 4 | O′ 6 | | O′ 18 | O′ 5 |
| O′ 4 | B 5 | | B 4 | O′ 9 |
| O′ 15 | B 7 | | B 6* | O′ 11 |
| O′ 16 | B 8* | | B 8 | O′ 14 |
| O′ 18 | | | | O′ 18* |
| O′ 19 | | | | B 2 |

* Phenogroups of relatively high frequency.

TABLE 7

*The inheritance of B, R–O and X–Z phenogroups of ram 5020T (Targhee) by 13 of his offspring*

| Offspring number | Phenogroups from sire in blood-group system | | |
|---|---|---|---|
| | B | R–O | X–Z |
| 6 | Q1 | * | X |
| 11 | Q1 | * | X |
| 14 | Q1 | O | Z |
| 15 | Q1 | R | Z |
| 19 | Q1 | R | Z |
| 27 | Q1 | R | X |
| 38 | Q1 | O | Z |
| 39 | Q1 | R | X |
| 49 | Q1 | O | X |
| 67 | Q1 | O | * |
| 33 | I5 | O | X |
| 50 | I5 | O | * |
| 66 | I5 | R | * |

* Contribution of sire not determined.

frequent in the flocks studied may not necessarily designate those which are frequent in the breed as a whole inasmuch as the blood samples which were tested were collected from a small number of flocks.

*Tests for independence of the blood-group systems B, R–O and X–Z:* Table 7 shows the B phenogroups, I 5 and Q 1, the R–O phenogroups, R and O (STORMONT 1951; RENDEL, NEIMANN-SØRENSEN and IRWIN 1954), and the X–Z phenogroups, X and Z, which 13 lambs inherited from their sire, Targhee ram 5020T. (The asterisks in Table 7 indicate that the contribution of ram 5020T to his offspring could not be determined. This was the case if in the R–O system, the sire, dam and lamb were all of group R, and in the X–Z system, if the lamb and its parents were all of type XZ.) These and other data indicate that the B, R–O and X–Z blood groups represent three different systems. While the genes controlling these three systems appear to assort independently, the possibility of loose linkage is not excluded by the present data.

## DISCUSSION

Observations of tests of cattle red cells with ovine isoimmune antisera (STORMONT, SUZUKI and RASMUSEN 1957) provided serological evidence for the existence in sheep (*Ovis aries*) of a blood-group system comparable to the complex B system of cattle (*Bos taurus*). Twenty-nine of 47 ovine isoimmune antisera tested contained antibodies which distinguished individual differences in cattle. From analysis of the cross-reactions of 20 of these 29 antisera, it was clearly evident that the reactions of 18 of the 20 antisera could be ascribed solely to phenogroups in the B system of cattle. Furthermore, it was possible to prepare reagents from some of these antisera which produced reactions in the cattle tests that paralleled the reactions of such previously known cattle reagents as I and $E'_3$. (Many of the cross-reactions of ovine isoimmune antibodies with B phenogroups of cattle were, however, unique patterns not previously encountered in the cattle studies). Also, as already noted, nine of the 26 reagents used in characterizing phenogroups in the B system of sheep were derived by absorbing bovine isoimmune antisera with selected sheep bloods. The specific antibodies in these nine reagents used for typing sheep red cells were known to have been engendered by B phenogroups in cattle. In addition the linear and nonlinear subtyping relationships observed in tests with the various reagents, regardless of their source and derivation (bovine or ovine isoimmune antisera or ovine heteroimmune antisera against cattle red cells), indicate that these reagents must be reacting in a single system in sheep.

The genetic data presented in this report provide confirmation of the earlier serological evidence for a blood-group system in sheep analogous to or homologous with the B system of cattle. Furthermore, the present evidence indicates that the B system of sheep may be fully as complex as its homologue in cattle. The question remains whether the demonstrated serological homology between B phenogroups in the two species extends to the level of the gene. It seems likely that it does, but in the absence of any authentic reports of the existence of viable hybrids between the two species, no means of obtaining direct evidence is presently available.

In genetic studies based on reactions of blood-typing antibodies, it may seem desirable to have reagents which react to produce rapid, complete hemolysis with all bloods with which they react. With such reagents there would be little question in differentiating negative from positive reactions. A reagent which produces a graded series of reactions from rapid, complete hemolysis through varying degrees of hemolysis to no hemolysis presents serious problems in interpretation in the absence of any knowledge of the phenogroups with which it cross reacts. Is the reaction due to a single population of antibodies in the reagent, or is it not? Resort to absorption with the red cells showing dubious reactions often leads to dubious results; manipulation of the ratio of cells to serum, the dilution of the serum, the number and length of absorptions and the temperature at which they are performed may result in removal of the antibodies or lowering their titer so that the reagent fails to react. Or, the antibodies may be only partially removed, and some cross-reactions are observed with the absorbed reagent. Indeed, many of the reagents, especially those prepared from ovine antisera, both isoimmune and heteroimmune, had low titers of antibodies, exhibited graded series of cross-reactions, and absorptions sometimes yielded dubious results. Nevertheless, it was just such reagents which ultimately proved to be most useful in diagnosing B phenogroups. The degree of cross-reaction with red cells of the same phenogroup formula was remarkably constant; the main difficulty became one of terminology.

Studies of the specificity of antigens and antibodies (reviewed by LANDSTEINER 1945) have shown that a single antigen may stimulate the production of multiple antibodies differing in specificity and in degree of affinity with the homologous antigen and closely related chemical substances. Such studies provide models for the cross-reactions in the B system of cattle (STORMONT *et al.* 1951), in the B system of sheep presented in this report, and for the cross-reactions of ovine and bovine isoimmune antisera in these systems (STORMONT *et al.* 1957). They have also been used as models for cross-reactions in complex blood-group systems in man (WIENER and WEXLER 1952) and in chickens (BRILES, IRWIN and MCGIBBON 1950). In the author's opinion, any attempt to describe the reactions of B phenogroups of sheep in terms of models involving discrete blood factors controlled by closely linked genes, subgenes, pseudoalleles or other hypothetical entities, rather than in terms of cross-reactions exhibited by a series of closely related antigens controlled by the alleles at a single complex locus would not only be a misrepresentation of the observations but would also serve to complicate interpretation of the reactions observed.

A reagent may show clear-cut individual differences in reactions with certain B phenogroups, but may only slowly and partially lyse bloods of other B phenogroups. The use of letters to signify cross-reactions with the individual reagents is helpful to visualize the cross-reactions of the phenogroups, but does involve the possibility of conveying the impression of an all-or-none situation, which is not the case in the B system of sheep or any system of comparable complexity in other species. Only by a consideration of the reactions with the entire battery of B-system reagents can the pattern of cross-reactions of the phenogroups be

determined. The reagents used in this study are certainly not the only B-system reagents which could be developed. In addition to the named reagents, other reagents clearly reacted in the B system, but were not given letter designations since including them in the pattern of cross-reactions would only serve to complicate an already complex nomenclature and would not further differentiate the 52 B phenogroups of this study. They were sometimes of use, however, in differentiating between certain combinations of B phenogroups, just as certain reagents of ovine isoimmune origin are of use in B phenogrouping in cattle (STORMONT 1958).

The decision to include or not include the name of any given reagent in the pattern of cross-reactions was sometimes difficult and arbitrary. A table of cross-reactions such as Table 4 in this report is especially desirable in detecting new phenogroups and as a guide in recognizing those already known. It is readily expandable; a new reagent may be designated with a new letter and its reactions then added to the table. The most serious difficulty which such a method presents as a basis for the nomenclature of a blood-group system is that it cannot be used readily by other investigators using a different battery of reagents. The coding system proposed in this report provides a system for naming phenogroups which is based on a few key reagents. New phenogroups can be readily fit into this nomenclature, and the sets of phenogroups, though somewhat arbitrary, appear to be reasonably logical groupings which can be expanded if necessary.

The coding system is less cumbersome than the use of a complex pattern of cross-reactions, and provides a simpler nomenclature with which to identify the phenogroups. Neither system is without defects, but difficulties which are likely to be encountered in duplicating many of the B-system reagents make exclusive use of the complete pattern of cross-reactions undesirable as the sole basis for describing the B system of sheep.

The coding system may also be used as a basis for naming the alleles in the B series, on the assumption that each phenogroup is the product of an individual allele. *B* is the base symbol for the locus, and the alleles are designated by appropriate superscripts: $B^{E'1}$ through $B^{E'6}$, $B^{I1}$ through $B^{I9}$, $B^{Q1}$ through $B^{Q10}$, $B^{O'1}$ through $B^{O'19}$ and $B^{B1}$ through $B^{B8}$, making a total of 52 alleles.

## SUMMARY

Evidence is presented for a blood-group system in sheep, the B system, which is serologically homologous to the complex B system in cattle and resembles the cattle system in its genetic features. It seems probable that the homology between the B systems in cattle and in sheep extends to the level of the gene or locus. Of a total of 50 reagents used in blood typing of sheep, 33 reacted in the B system. Tests with these reagents combined with genetic data indicate a minimum of 52 alleles in the B series of sheep.

ACKNOWLEDGMENT

The author wishes to thank DR. CLYDE STORMONT for his helpful suggestions during the progress of this study and in the preparation of the manuscript, and MISS YOSHIKO SUZUKI for her valuable assistance with much of the laboratory work. The blood samples were obtained by the cooperation of MESSRS. FRED GROVERMAN, ROBERT FINLEY, WENDELL KYLE, DONALD PRICE, and CLAIR TERRILL.

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# ELECTROPHORETIC AND IMMUNOCHEMICAL STUDIES WITH ENDOSPERM PROTEINS OF MAIZE MUTANTS

DREW SCHWARTZ

*Biology Division, Oak Ridge National Laboratory,*[1] *Oak Ridge, Tennessee*

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IN an effort to narrow the gap between the primary action of the gene and the phenotypic modification by which the gene is recognized, we have initiated a study of gene-induced protein differences in maize using electrophoretic and immunochemical techniques. Specifically, we are investigating the enzymes involved in starch synthesis and degradation in the maize endosperm tissue (SCHWARTZ 1959). This material is well suited for such studies. It has been subjected to extensive cytogenetic analysis, and many genes are known and available that affect starch synthesis. The endosperm is the site of concentrated enzymatic activity almost entirely devoted to synthesis of starch. In fact, electrophoretic analysis is possible with extracts from single endosperms. Many of the nonallelic mutant genes that block starch synthesis give rise to quite similar mutant phenotypes. This paper deals mainly with the effect of the *sh*$_1$ mutant on chromosome 9.

## METHODS AND MATERIAL

Immature maize endosperms are used in these studies. The ears were harvested 16 days after fertilization, near the peak of starch synthesis. Kernels were cut off the cobs and stored in plastic bags at —20°F. The material was macerated and centrifuged at high speed (100,000 × g) to remove cell debris and particulate fractions, and the supernatant was used for the electrophoretic separations. Since no differences were observed in the electrophoretic patterns when the kernels were macerated with or without the tiny embryo, we did not bother with the tedious task of removing the embryo in subsequent runs.

Electrophoretic separations were made on both crude and concentrated extracts. The extracts were concentrated by pressure dialysis, dialysis against 20 M Carbowax (KOHN 1959), or by lyophilization.

When the unconcentrated extracts were used, they were applied to thick pieces of filter paper for insertion into the starch gel so that a fairly large amount of solution could be applied. Whatman No. 1 filter paper was used for the concentrated extracts. The Smithies technique for starch gel electrophoresis and amido black staining was used (SMITHIES 1955). Best separations were obtained by use of borate buffer at *p*H 8.5 (0.1 $\mu$ in starch gel, 1.0 $\mu$ in electrode trays). The runs were of about 4-hour duration at room temperature with a voltage drop of 7 v/cm along the starch gel. For accurate comparisons of electrophoretic patterns of the

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

various mutants tested, two runs were made side by side on the same gel by applying the extracts at each end of the piece of filter paper to be inserted in the starch gel so that the extracts overlapped in the center. The best patterns were obtained with the material concentrated by lyophilization. A Spinco Model H electrophoresis-diffusion instrument was used for the moving boundary electrophoretic study with a phosphate buffer at *p*H 7.5 and 0.1 $\mu$.

The immunochemical analysis was made by the Ouchterlony double diffusion technique (OUCHTERLONY 1948). Antiserum reagents were prepared in rabbits by injecting the 100,000 × g supernatant of endosperm extracts with Freund's technique (water in oil emulsion).

## RESULTS AND DISCUSSION

We have not as yet succeeded in separating the endosperm proteins with agar gel (veronal buffer *p*H 8.6, 0.02 $\mu$) or paper (veronal buffer *p*H 8.6, 0.075 $\mu$) electrophoresis. Neither of these techniques gives clear separation into discrete bands. In starch gel, however, excellent separation is obtained, with the proteins moving as sharp discrete bands (Figure 1a). In this technique the separation of some of the endosperm proteins may be influenced by their relative affinity for the starch gel. It is especially true for the enzymes involved in the synthesis and degradation of starch. This is illustrated by two-dimensional electrophoresis of the crude extract, as for example, paper followed by starch gel. The protein showing the fastest migration rate on paper appears as the slowest moving band in the starch gel. By this technique, it is possible to determine the position of the protein bands in paper electrophoresis, and it was found that the proteins move

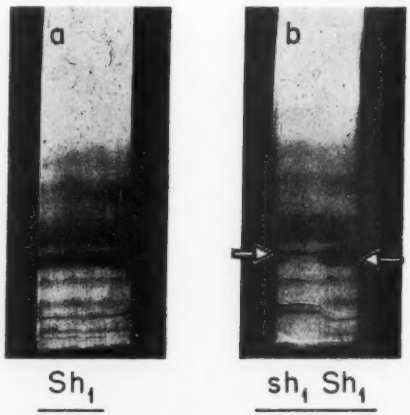


FIGURE 1a.—Starch gel electrophoresis of nonmutant endosperm extract (W-23).

FIGURE 1b.—Starch gel electrophoresis pattern of $Sh_1/Sh_1/Sh_1$ and $sh_1/sh_1/sh_1$ endosperm extracts run side by side on the same gel. The arrow on the right points to the $Sh_1$ protein. The arrow on the left points to the forward moving band (see text).

as broad smears as opposed to the discrete bands on the starch gel. The four slowest moving bands have properties characteristic of amylases. They are very active in reducing the size of the starch polymers. This can be shown directly by staining the gel with iodine after a short period of incubation. The gel as a whole stains blue while four red bands appear at the exact positions of the four slow-moving protein bands. This comparison is made by slicing the gel in half to present two identical faces, one of which is stained with amido black and the other with iodine. It is of interest that these amylases are found in tissue involved with synthesis of starch and not degradation; they may also play some role in the synthetic processes, possibly in primer formation. Since these enzyme bands are identified only by the iodine starch test, they may also include the "Q" or branching enzyme.

In this paper, attention will be focused on the heavy staining band seen in Figure 1a. This protein is not an amylase but has been found to show alkaline phosphatase activity by the application of the histochemical method of GOMORI (1952) to zone electrophoresis in starch gel as modified by POPP (personal communication). We will present evidence to show that the synthesis of this protein band is under the control of the *Sh*$_1$ gene on chromosome 9 and, for convenience, it will be designated as the Sh$_1$ band, although other genes may also be involved.

In all, about 30 different genetic stocks (obtained from Maize Cooperation) homozygous recessive for various endosperm starch mutants were tested. These included *su*$_1$, *su*$_1^{am}$, *su*$_2$, *bt*$_1$, *bt*$_2$, *sh*$_1$, *sh*$_2$, *sh*$_3$, *sh*$_4$, *ae*, *h*, *o*$_1$, *o*$_2$, *wx*, *wx*$^a$, *du*$_1$, *fl*$_1$, *fl*$_2$, singly as well as a number of double mutant combinations. Three of the stocks tested were homozygous recessive for the *sh*$_1$ gene, and these lacked the Sh$_1$ band completely. All the other stocks carried the *Sh*$_1$ allele and showed the presence of the Sh$_1$ band in the electrophoretic separation on the starch gel. Figure 1b shows the starch gel pattern obtained when extracts from *Sh*$_1$/*Sh*$_1$/*Sh*$_1$ and *sh*$_1$/*sh*$_1$/*sh*$_1$ endosperms were run side by side on the same starch gel.

The absence of a major protein component in *sh*$_1$/*sh*$_1$/*sh*$_1$ endosperm is also seen in moving boundary electrophoresis patterns (Figure 2).

The intensity of the Sh$_1$ protein band is gene dose dependent. Since maize endosperm is triploid, receiving two nuclei from the female and one from the male, it was possible to compare the four genotypes, *Sh*$_1$/*Sh*$_1$/*Sh*$_1$, *Sh*$_1$/*Sh*$_1$/*sh*$_1$, *Sh*$_1$/*sh*$_1$/*sh*$_1$, and *sh*$_1$/*sh*$_1$/*sh*$_1$. For these comparisons the two heterozygous combinations were made from reciprocal crosses in which the same two homozygous plants were used as both male and female parents. The intensity of the Sh$_1$ band increased with increasing doses of the *Sh*$_1$ gene.

The correlation between the amount of Sh$_1$ protein produced in the endosperm (as determined by staining intensity) and the dose of the *Sh*$_1$ gene was confirmed by the immunochemical studies.

A number of precipitation lines appear in the Ouchterlony double diffusion test when antiserum containing antibodies specific for the endosperm proteins is allowed to diffuse against the endosperm extracts. The heaviest of the precipitation lines involves an antigen found only in the *Sh*$_1$ kernels. All the *Sh*$_1$ stocks

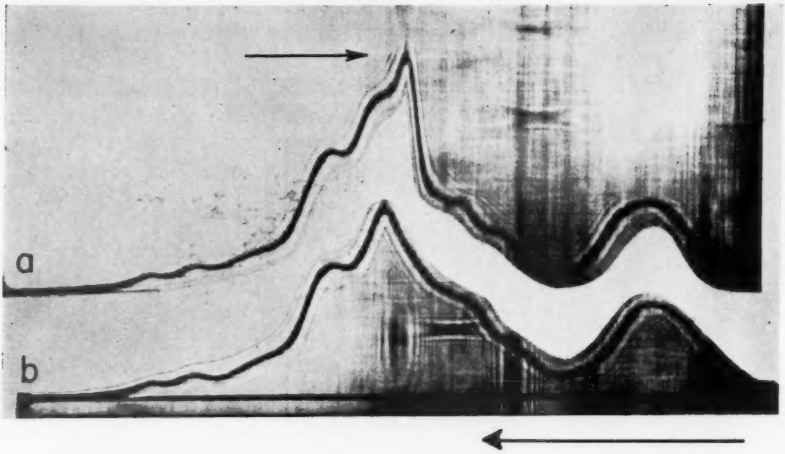


FIGURE 2.—Moving boundary electrophoresis patterns of crude (a) $Sh_1/Sh_1/Sh_1$ and (b) $sh_1/sh_1/sh_1$ extracts showing an extra peak in (a).

were tested in this manner and shown to form this precipitation band. The three $sh_1$ stocks lacked this precipitation band completely when tested against antiserum from rabbits immunized against $Sh_1$ endosperm extracts. This is an extremely sensitive test for the presence of an antigenic protein. A concentration of antigen insufficient to form a detectable precipitation band can still be detected since it would cause a bending in the precipitation band formed by the antigen in an adjacent well. Extracts from the $sh_1/sh_1/sh_1$ plants caused no such bending (Figure 3).

By the technique of immunoelectrophoresis it was possible to establish that the $Sh_1$ antigen missing in the $sh_1$ extracts is identical to the $Sh_1$ protein in the electrophoretic separation. Endosperm extracts were separated electrophoretically, and the starch gel was sliced in two. One slice was stained to determine the exact positions of the protein bands; the other was placed in an agar plate in which a section of the agar gel had been cut out to receive the starch gel slice. The antiserum was put into a trough running parallel to the entire length of the starch gel. A strong precipitation band was formed around the position of the $Sh_1$ protein band in the starch gel when extracts from $Sh_1$ endosperms were used. No such precipitation band appeared when the $sh_1$ extract was tested in this manner (Figure 4).

In a similar experiment, the section of the starch gel containing the $Sh_1$ protein band was cut out and placed in a three-well agar plate. The other two wells were filled with the antiserum and the crude extract from $Sh_1$ endosperms. The precipitation band formed between the section of starch gel and the antiserum coalesced with the heavy precipitation band formed between the extract and the

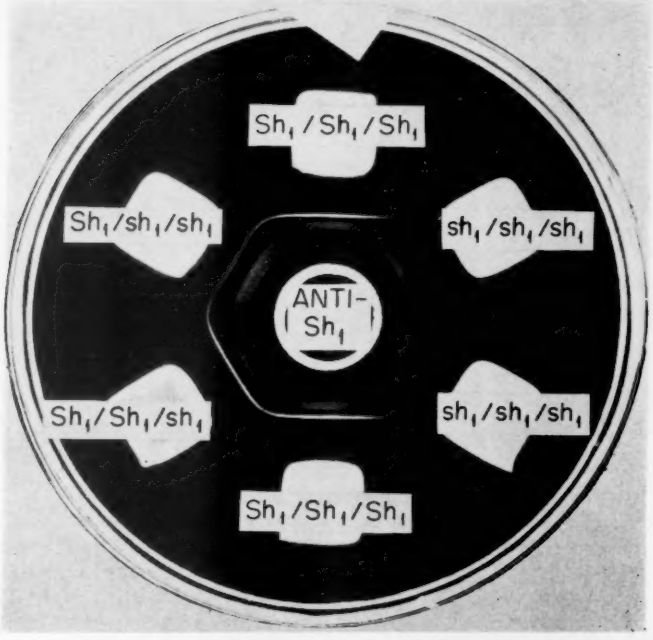


FIGURE 3.—Ouchterlony precipitation patterns found by allowing endosperm extracts to diffuse against antiserum of rabbits immunized against $Sh_1/Sh_1/Sh_1$ endosperm. The antiserum is in the center well and the extracts are in the surrounding wells.

antiserum (i.e., the band that is missing in the $sh_1$ material), thus establishing the identity of the protein and antigen.

In the Ouchterlony test, the relative concentration of antigen in a number of extracts can be compared, the intensity of the precipitation band and its position being used as criteria. Such an analysis of the two homozygous and two heterozygous genotypes confirmed the electrophoretic results indicating that the concentration of the $Sh_1$ antigen is gene dose dependent (Figure 3).

In the previously described experiment, in which the starch gel slice was used in the immunoelectrophoresis test, a continuous precipitation band was found along the entire length of the starch gel slice (see Figure 4). Further investigation established that this precipitation band is caused by dextrins diffusing out of the starch gel and precipitating with the antiserum. This precipitation band appeared even when a starch gel to which no endosperm extract had been added was used. In fact, dextrins were agglutinated by nonimmunized rabbit sera. Whether this involves a nonspecific agglutinization or these animals normally contain antibodies against the dextrins has not as yet been established.

Further proof that the $Sh_1$ protein band is under the control of the $Sh_1$ gene

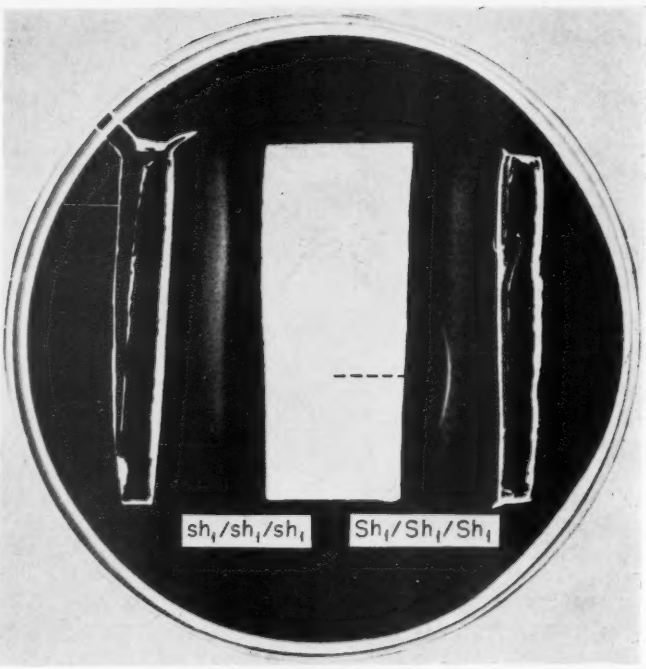


FIGURE 4.—Immunoelectrophoresis of $Sh_1/Sh_1/Sh_1$ and $sh_1/sh_1/sh_1$ endosperm extracts using anti-$Sh_1$ rabbit serum. The two extracts were run side by side on the same gel. The dotted line in the gel indicates the position of the $Sh_1$ protein band as determined by staining the other half of the starch gel.

was obtained from a study of Dissociation (*Ds*) suppression of this gene. MCCLINTOCK (1951) has described a transposable element *Ds* that, when situated in the close proximity of a particular gene, can completely suppress the action of the gene so that, in the homozygous condition or heterozygous with a recessive, the completely recessive phenotype is produced. *Ds* is completely stable in the absence of Activator (*Ac*). In its presence, however, *Ds* is frequently removed from its site next to the gene by either chromosome breakage or transposition. As a result of such events, the dominant phenotype is restored.

We have tested four independently arising cases of $Sh_1$ suppression by *Ds*. This material was kindly supplied by DR. MCCLINTOCK. One of these lines was homozygous $DsSh_1$; the other three were heterozygous with the recessive $sh_1$ allele. Endosperm extracts from all four lines were similar to homozygous $sh_1$ material in that, when *Ac* was absent, the $Sh_1$ protein-antigen was completely lacking (Figure 5a). These were tested both electrophoretically on starch gel and immunochemically. When the same genotypes were tested in the presence of *Ac*,

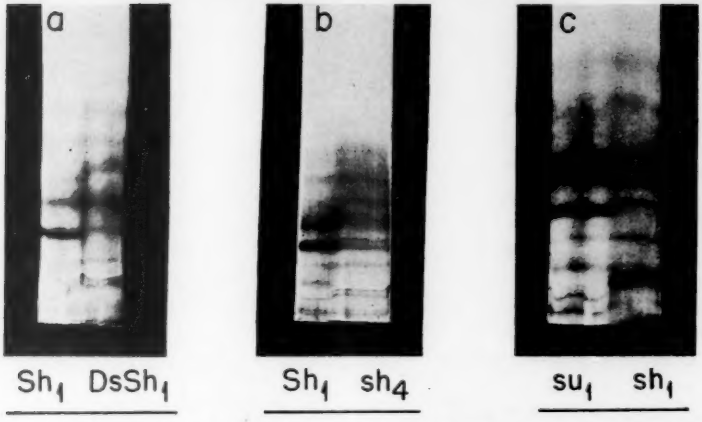


FIGURE 5a.—Starch gel electrophoresis pattern of $Sh_1/Sh_1/Sh_1$ run alongside $DsSh_1/DsSh_1/DsSh_1$.

FIGURE 5b.—Starch gel electrophoresis pattern of $Sh_1/Sh_1/Sh_1$ run alongside $sh_4/sh_4/sh_4$.

FIGURE 5c.—Starch gel electrophoresis pattern of $su_1/su_1/su_1$, $Sh_1/Sh_1/Sh_1$ run alongside $Su_1/Su_1/Su_1$, $sh_1/sh_1/sh_1$.

the $Sh_1$ protein-antigen was found to be present, but in somewhat reduced amount. This reduction is expected since the kernels are mosaic for shrunken and nonshrunken tissue.

All of the tested $Sh_1/Sh_1/Sh_1$ material recessive for the other endosperm mutants showed the presence of a high concentration of the $Sh_1$ protein, with the exception of the $sh_4$ mutants. Two $Sh_1/Sh_1/Sh_1$, $sh_4/sh_4/sh_4$ strains in different backgrounds were tested, and both showed a drastic reduction in the concentration of the $Sh_1$ protein as determined both on starch gel electrophoresis by the intensity of the stained band and immunochemically by the intensity and position of the precipitation band in the Ouchterlony test. Though less intense, the $Sh_1$ band in the $sh_4$ endosperm extracts migrated at the same rate as this band in the other endosperms (Figure 5b).

The results reported in this paper clearly establish that the $Sh_1$ gene is involved in the control of the synthesis of a major protein component in the endosperm. This component has not been found in germinating seed or in immature or mature pollen. The question remains as to whether this protein is not synthesized at all in the $sh_1$ mutant endosperm or is synthesized but modified in such a manner as to be noncross-reacting immunologically with the protein produced in the presence of the $Sh_1$ allele and to have an altered mobility in starch gel electrophoresis. The presence of a new antigen in the $sh_1$ extracts could not be demonstrated. Comparison of the starch gel patterns of $Sh_1$ and $sh_1$ extracts failed to reveal the presence of a band in $sh_1$ that was not also present in the $Sh_1$ extracts. The only difference observed in $sh_1$ material, other than the absence of the $Sh_1$ protein, was an increase in the intensity of the band that migrates just ahead of the $Sh_1$ band

(Figure 1a). In normal nonmutant material this forward band is quite weak and stains very lightly. That the increase in intensity of the forward band is under the control of the *$sh_1$* gene and not simply associated with a decrease in the concentration of the $Sh_1$ protein can be shown by comparing starch gel patterns of *$sh_1/sh_1/sh_1$* and *$DsSh_1/DsSh_1/DsSh_1$* endosperm extracts. Both of these completely lack the $Sh_1$ protein band. If the increased intensity of the forward band is a consequence of a reduction of the $Sh_1$ protein, then both extracts should show the forward band with about equal intensity. However, the homozygous *$DsSh_1$* endosperms show only a very weak band at the forward position, similar to the band in the *$Sh_1$* homozygotes. It thus seems that the *$sh_1$* allele is not an amorph, although the connection between the elimination of one protein and intensification of a second is not clear. The possibility also exists that the more intense forward moving band in the *$sh_1$* material represents a different protein but one that has the same migration rate on the starch gel as that occurring in the extracts from the *$Sh_1$* plants.

The forward moving band is of much interest and is being intensively investigated. This band is not seen in endosperms of at least three mutants (*$su_1$*, *$sh_2$*, and *$sh_3$*) (Figure 5c). Tests of the various double mutant combinations point to genic interaction in the control of this protein, similar to that described by KRAMER, PFAHLER and WHISTLER (1958) on the phenotype level. This band shows a faster migration rate in *$sh_4/sh_4/sh_4$* endosperms.

The effect of the *$sh_1$* mutant in maize appears to be similar to that of the *$td_1$* mutant in Neurospora (SUSKIND, YANOFSKY, and BONNER 1955) in that it results in the complete absence of a protein produced in nonmutant material without the associated appearance of another cross-reacting protein. We are in the process of surveying endosperm mutants in a search for plants that contain the $Sh_1$ protein with an altered rate of migration.

## SUMMARY

Electrophoretic and immunochemical investigations on endosperm proteins in maize mutants that affect starch synthesis are described. The *$Sh_1$* gene controls the formation of a major protein component in the endosperm that is completely lacking in *$sh_1/sh_1/sh_1$* material. The suppression of *$Sh_1$* by *Ds* also results in the absence of this protein. Removal of *Ds* from its position adjacent to the *$Sh_1$* gene under the influence of *Ac* causes a reappearance of the $Sh_1$ protein. No evidence was found for the synthesis of an altered protein by the *$sh_1$* mutant; however, the mutant gene increases the intensity of another protein band that is normally present in nonmutant endosperms.

## ACKNOWLEDGMENT

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# THE CONTROLLING ELEMENT IN SEX CHROMOSOME BEHAVIOR IN SCIARA[1]

HELEN V. CROUSE

*Department of Botany, Columbia University, New York, New York*

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GENETIC and cytological studies have been made on a number of species of Sciara (see METZ 1938 for review). Common to all of these species is a series of unusual and remarkable cytological phenomena which involve the sex chromosome and which are related to the differentiation of sex in this genus. The first of these is encountered at the second meiotic division in the male, when, following the selective elimination of paternal homologues at the first spermatocyte division, the maternally derived X chromosome undergoes equational nondisjunction. Thereby, instead of a haploid set of chromosomes, such as the egg transmits (three autosomes and one X), the sperm nucleus comes to include two identical X's and three autosomes. Although the sperm transmits two X chromosomes to the zygote, in no cell of the developing embryo are both of them retained permanently: one is eliminated from the somatic nuclei of the females, both from the somatic nuclei of males, and one from the germ cells of each sex.

This pattern of sex chromosome behavior constitutes a unique sex determining mechanism. All zygotes begin development under the influence of the same chromosome complement, three X's and three pairs of autosomes. A differential between male and female is not established until anaphase of the seventh or eighth cleavage—and then only in the somatic nuclei—at which time there is an elimination of one or of both paternal X's in the production of female and male embryos, respectively (DU BOIS 1933). The germ line retains all three sex chromosomes until the germ cells have migrated to the definitive gonad site; then, in both sexes alike, one paternal X is eliminated. This elimination occurs not at anaphase but in resting cells at a stage when the chromosomes are in the form of fairly compact prochromosomes; one of the paternal X's appears to migrate directly through the nuclear membrane into the cytoplasm and subsequently degenerates (BERRY 1941).

This series of interrelated and unusual phenomena demonstrates that the cells of the developing Sciara embryo can distinguish between sex chromosomes and autosomes, between maternal and paternal homologues. The nature of this "recognition" of chromosomes is not understood. One of the first questions to be answered, however, and one which is experimentally feasible, is whether a local-

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ized segment on the sex chromosome, or the chromosome as a whole, is involved in the process.

With this question in mind, a number of reciprocal translocations between chromosome X and the autosomes have been obtained and a study made of their inheritance and their cytological behavior during spermatogenesis (CROUSE 1943, 1960). The purpose of the present paper is to describe a new translocation in *S. coprophila*, designated "Oak Ridge T1," with which it has been possible to identify a proximal segment of heterochromatin on the X as the essential element controlling the unique behavior of this chromosome. The effects produced by the new translocation will be compared and contrasted to effects produced by a series of X translocations in the same species.

## MATERIALS AND METHODS

The translocations were induced by X-ray irradiation (4000r) of sperm from monogenic strains of *S. coprophila* obtained originally from PROFESSOR METZ's

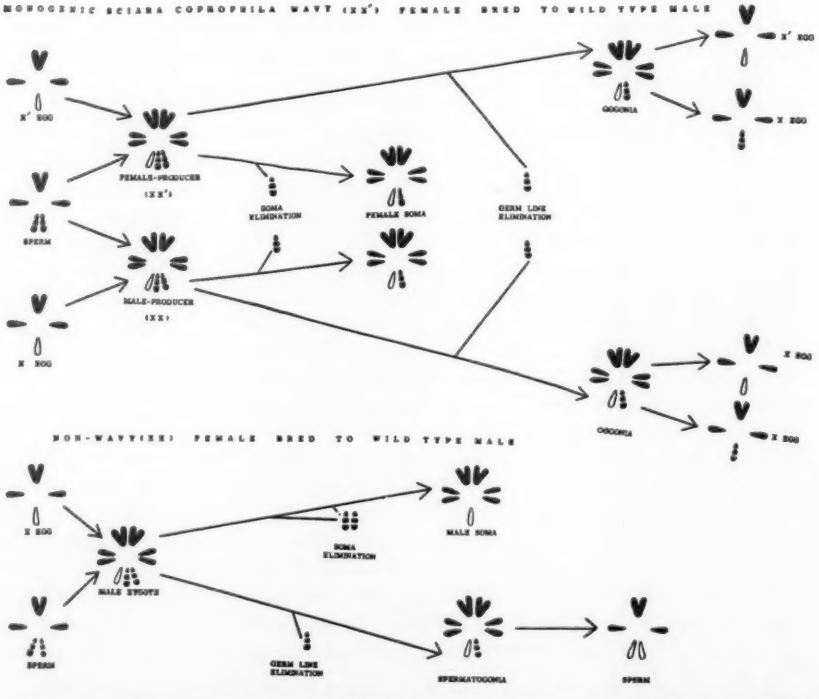



FIGURE 1.—Diagram illustrating the history of chromosomes in monogenic *S. coprophila* when Wavy females (XX') and non-Wavy females (XX) are bred to wild type males. The limited chromosomes are omitted from consideration.

laboratory. Use was made of the available sex-linked markers, Wavy (*W*) and swollen (*sw*) and of several autosomal dominants. In contrast to digenic species of Sciara, in which single families consist of both sons and daughters, the females in monogenic lines are either female producers or male producers. The inheritance of sex of progeny is shown in Figure 1. Genetically there are two kinds of sex chromosomes, designated X and X′; females are either X′X (female producers) or XX (male producers), and males are normally XX (X0 in soma). Wavy is a dominant marker on X′, and swollen is a recessive on X. In the earlier experiments (CROUSE 1943) the translocations were detected by means of "linkage" of autosomal and sex-linked factors. Later a more useful criterion presented itself: the occurrence, among the progeny of females heterozygous for any one of the X translocations, of exceptional patroclinous sons or exceptional matroclinous daughters (see CROUSE 1960).

Once an X translocation had been detected and the fact established that it was transmissible through both the male and female germ line, the positions of breakage on the chromosomes were determined by salivary gland chromosome analysis, and cytological studies were made on spermatogenesis in males receiving the translocation from their mother. It should be recalled from the introductory statement to this paper that, unlike oögenesis which is perfectly orthodox in Sciara, spermatogenesis is very unusual: each primary spermatocyte gives rise to only one sperm by virtue of the unequal nature of both meiotic divisions. At the first division there is no synapsis or crossing over, and at anaphase a directed segregation of homologues occurs as the paternally derived set of chromosomes is cast away in a bud. At the second division the maternal homologues divide equationally, but, as pointed out above, the two halves of the X fail to separate and both become included in the sperm nucleus. Their fate at the time of chromosome elimination in the embryo has already been described; the somatic and germ line chromosome eliminations are indicated on the diagram in Figure 1. For cytological study of spermatogenesis aceto-orcein squashes of Carnoy-fixed pupal testes were used chiefly, but these were supplemented by Feulgen-stained sections of comparable material preserved with a variety of fixatives, including Kahle, San Felice, and Gilson's corrosive sublimate.

As soon as spermatogenesis had been studied cytologically for a given translocation, the chromosome constitution of the sperm was known. With this knowledge it was possible to learn how the translocation chromosomes behaved at the "critical" stages in the embryo without the exacting cytological task of observing the actual chromosome eliminations: for, upon union of the "known" sperm with eggs produced by normal females, the nature of the somatic and germ line eliminations could be *deduced*, provided a determination was made of the somatic (salivary gland) complement and the germ line constitution (both genetic and cytological studies) of these individuals. Such genetic and cytological studies were made for nine different X translocations. It is not the purpose of this paper to present all of these data; some of the results have already been published, and others are in the process of publication. Included in the present paper are selected data which serve to show that the proximal mass of heterochromatin on the

X is the essential controlling element in the extraordinary behavior of this chromosome.

## RESULTS

*The chromosomes in the translocation material:* Shown on the diagram in Figure 2 are the important structural features of the X chromosome of *S. coprophila* as observed in the salivary gland nuclei. The 36 units of length assigned to it are based on the published maps (CROUSE 1943). There are 12 map sections, each with subdivisions A, B, and C. The photomicrograph in Figure 3 shows the entire salivary complement. The exact location of centromeres on these chromosomes cannot be determined by microscopic study because there is no chromocenter, and the centric regions are not characterized by any visible differential quality. By determining the positions of chromosome breakage in 18 different translocations and the metaphsae configuration for certain of these, it was possible to deduce that salivary element IV corresponds to the only V-shaped chromosome of the complement and that the proximal ends of the three remaining chromosomes are as indicated in Figure 3a (CROUSE 1943 and unpublished). Moreover, it has been possible to localize fairly accurately the X centromere in map region 12C immediately adjacent to the large mass of heterochromatin which terminates this map section and which likewise constitutes the end of the chromosome. Of key significance in this localization were translocation "Oak Ridge T1" and a long paracentric inversion on the X. The paracentric nature of the inversion was established by cytological studies (unpublished) on oögenesis comparable to those made by CARSON on *S. impatiens* (CARSON 1946). In addition to the centromere and terminal mass of heterochromatin, a third structural feature is indicated on the diagram, namely, three repeats located in map regions 2C, 10B, and 12B. These repeats are very short, each composed of a doublet and a singlet. Usually in smear preparations the interstitial one (10B) does not reveal itself through lateral synapsis (see Figure 3). It is not that this repeat fails to undergo synapsis with the other two, but that in so doing the chromosome loop produced by the segment 10B-12B is so short that the chromosome breaks in this region or the lateral attachment is ruptured. All three repeats are laterally synapsed in the cell shown in Figure 4. Indicated by means of arrows on the diagram are the positions of breakage for each of the nine translocations under

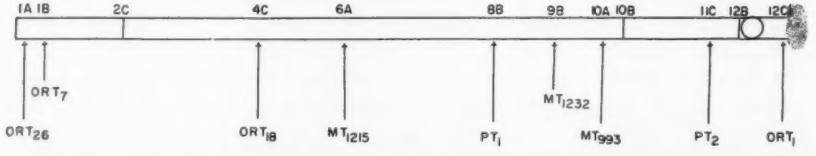



FIGURE 2.—Diagrammatic representation of the salivary X chromosome of *S. coprophila*. The centromere is shown as a circle, the terminal heterochromatin as a stippled mass. The positions of the three repeats are indicated with a vertical line and the positions of breakage for the nine translocations by arrows; the positions are given in terms of the salivary maps.

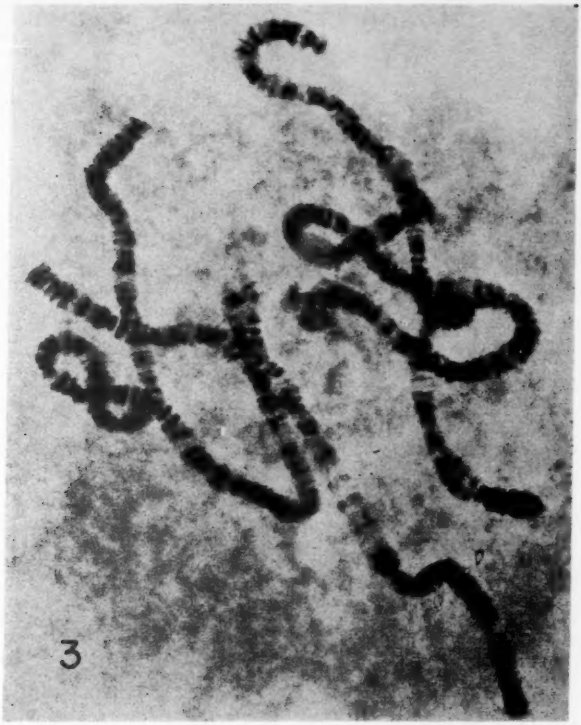
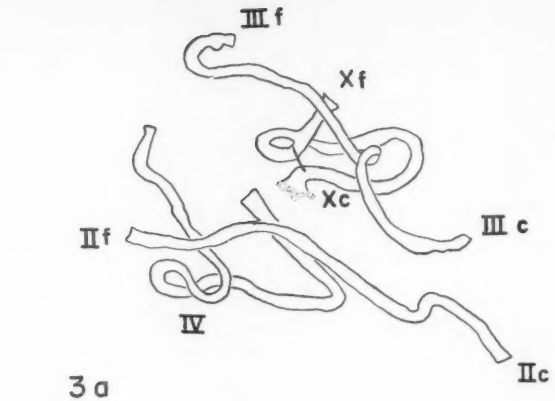


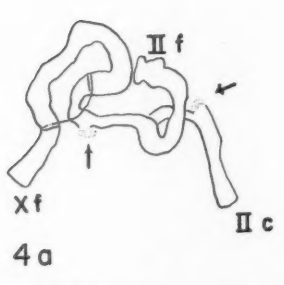

FIGURE 3.—Photomicrograph of the normal salivary complement of *S. coprophila.* × 600.

study. The letters M, P, and OR designate Missouri, Pennsylvania, and Oak Ridge, respectively. Thus, "OR T1" means the first of a series of X translocations obtained at the Oak Ridge Laboratory, etc.

*The spermatogenesis studies:* The results of the cytological studies on spermatogenesis are summarized in Table 1. In the second column of the table the autosome involved in each of the translocations is listed, together with the position of breakage according to the salivary maps. It will be noted that all of the translocations except M T1215 were simple, involving a single break on the X and a single break on one of the autosomes. Even M T1215 was simple as regards the X breakage, and only two translocation chromosomes composed partially of X were formed by the rearrangement. Thus, in all nine cases the X was distributed between two rearranged chromosomes.

In the third and fourth columns of the table there is presented the essential behavior of the rearranged chromosomes during the crucial secondary spermatocyte division. (For the sake of brevity in the table the chromosome observed to undergo equational nondisjunction is referred to as the "precocious chromo-



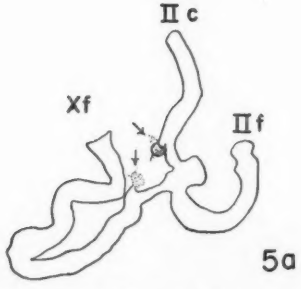




FIGURE 3a.—Outline drawing of the chromosomes shown in Figure 3. The proximal ends of the rods are indicated by the letter c, the distal ends by f. IV is the V-shaped chromosome.

FIGURE 4a.—Outline drawing of the chromosomes shown in Figure 4. The terminal heterochromatin of the X is stippled; the arrows indicate its position on the normal X and also on the translocation chromosome bearing the centromere of II.

FIGURE 5a.—Outline drawing of chromosomes shown in Figure 5.

some," the original terminology applied by METZ. For all nine of the translocations only one of the rearranged chromosomes underwent equational nondisjunction; the other chromosome underwent congression and equational division along with the autosomes. In seven of the nine cases the chromosome which displayed behavior normally characteristic of the X, i.e., equational nondisjunction, could be identified (see fifth column of the table); in all except OR T1 it was the translocation chromosome composed of a distal autosomal segment and a proximal segment of X including the X centromere and terminal heterochromatic mass.

In OR T1 the break on the X separated this terminal heterochromatin from the remainder of the chromosome. Thereby, following reciprocal exchange with the broken II, two translocation chromosomes were formed: one composed of a

TABLE 1

*Cytological studies of spermatogenesis*

| Translocation | Autosome and break position | Shape of "precocious" chr. | Shape of other translocation chromosome | Identification "precocious" chromosome |
|---|---|---|---|---|
| M T993 | II–18B | rod | rod | no |
| M T1215 | II–15C<br>IV–41B and 50A | rod | V-shaped | yes |
| M T1232 | III–25C | rod | rod | yes |
| P T1 | II–18B | rod | rod | no |
| P T2 | II–16C | rod | rod | yes |
| OR T1 | II–18C | rod | V-shaped | yes |
| OR T7 | IV–36B | rod | J-shaped | yes |
| OR T18 | IV–49C | rod | J-shaped | yes |
| OR T26 | IV–37B | rod | J-shaped | yes |

For explanation see text.

proximal segment of chromosome II attached to and ending in the X heterochromatin, the other composed of practically the entire X attached to a distal segment of chromosome II (see Figures 4, 4a, 5, and 5a). The former chromosome, of course, should be a tiny rod at metaphase and possess the centromere obtained from II; the latter should be a somewhat unequal V and possess the centromere obtained from the X.

That this is exactly what was found can be seen by studying the photomicrographs shown in Figures 6, 8, and 9. By way of comparison the normal chromosome group at second metaphase is shown in Figure 7. The cells in this figure show very clearly a rod-shaped "precocious" chromosome at one pole and five chromosomes on the plate. Of the five, three are members of the regular complement and two are V-shaped "limited" chromosomes. The limited chromosomes are supernumerary and occur only in the germ line; they are eliminated from the somatic nuclei of the embryo at the fifth or at the sixth cleavage (Du Bois 1933). In some species of Sciara limited chromosomes do not occur. Their occurrence in *S. coprophila* is of no significance for the problems under consideration in this paper. The spermatocytes from the OR T1 testis (Figures 6, 8, and 9) reveal a complement of four ordinary and three limited chromosomes (the number of limited chromosomes is variable in this species). All of the limited chromosomes are V-shaped. In the polar view of second metaphase shown in Figure 6 the four ordinary chromosomes lie adjacent to each other in the lower half of the nucleus, the three limited ones roughly in the upper half; the chromosomes at this stage are clearly double, and the tiny rod looks superficially like a small V; of the remaining three ordinary chromosomes only one is a rod, the other two being V-shaped. In other words, by means of translocation OR T1 a normal complement of three rods and one V was converted into two rods and two V's (see above). That this is clearly the case can be seen in the anaphase in Figure 9 taken from the same testis; the rod-shaped "precocious" chromosome is at the upper pole and looks somewhat like a triangle whose apex is pointing

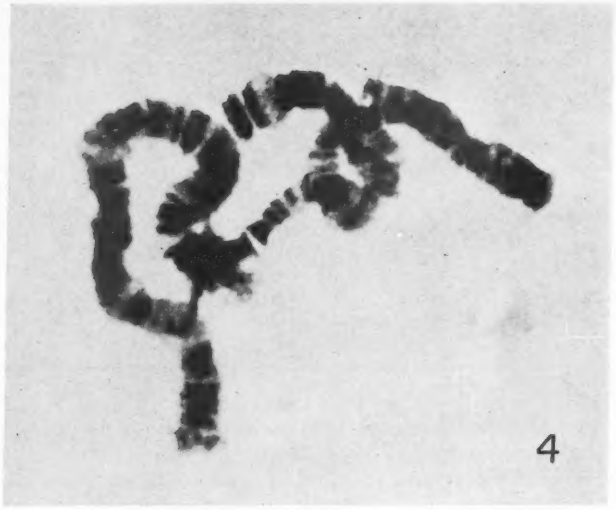


FIGURE 4.—Photomicrograph of heterozygous OR T1 showing lateral synapsis of the three repeat regions. × 1500.

FIGURE 5.—Photomicrograph of heterozygous OR T1. In this nucleus the interstitial repeat is not synapsed with the other two. × 1500.

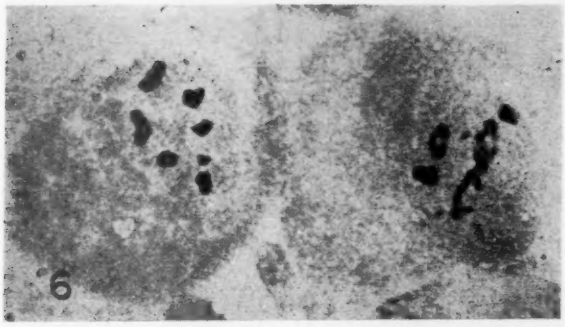


FIGURE 6.—Orcein squash of secondary spermatocytes of OR T1. Photomicrograph at 1500×. Polar view of M II; in the A II figure the tiny rod chromosome is dividing equationally (see text).

FIGURE 7.—Secondary spermatocytes of PT 1 showing a chromosome complement which is indistinguishable from that observed in normal material at this stage. Note the rod-like precocious chromosome and the two other rods on the equatorial plate (see text).

toward the equatorial plate; all the chromosomes undergoing division on the plate are V-shaped except one; this rod is III. In orcein squashes it is difficult to determine, in polar view of second metaphase, which chromosome is exhibiting precocity, e.g., the one in Figure 6; in side view, however, the "precocious" chromosome can be recognized readily, and in all four side views in Figure 8 as well as in the one in Figure 9, the tiny rod is behaving precociously. In all these figures this chromosome is pale and attenuated and definitely oriented with its apex towards the plate. Which end of the rod bears the centromere of II and

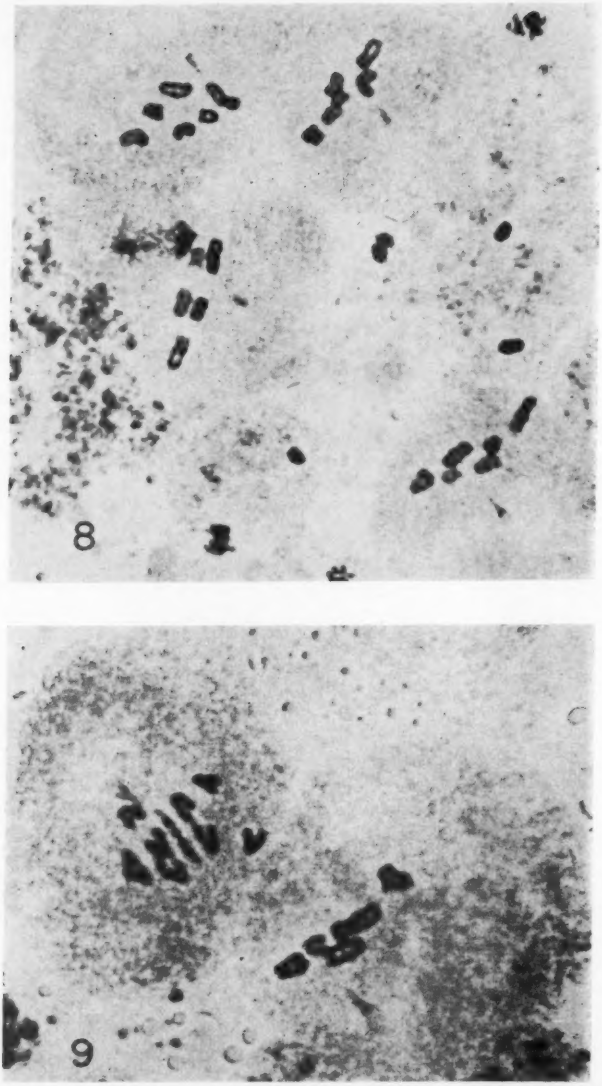



FIGURE 8.—Same testis as shown in Figure 6. Side views of M II in which precocious behavior of tiny rod can be clearly seen. × 1500.

FIGURE 9.—Same testis as shown in Figures 6 and 8. Side view of M II; A II figure shows precocious tiny rod towards upper pole. × 1500.

which the heterochromatin from the X is not certain. Very often in orcein squashes of normal material, particularly in *S. reynoldsi*, the two chromatids of the precocious chromosome diverge distally as they go to the pole. Reasoning by analogy, then, one would be led to believe that the centromere of the tiny rod in the OR T1 spermatocytes actually backs into the pole and that the end of this translocation chromosome which is made up of the X heterochromatin actually leads the way. Whether this is indeed the case has not yet been determined from the sectioned material; it is hoped that with osmium fixation, better presentation of the spindle fibers will be obtained.

The anaphase shown in Figure 6 is very interesting because the tiny rod has failed to exhibit precocity in this spermatocyte, and, instead, has clearly undergone equational division. The fate of such cells subsequent to second anaphase is unknown. An estimate of the frequency with which this behavior occurs was made by careful study and tabulation of approximately 1000 spermatocytes at second metaphase and anaphase: in 940 cells the tiny rod clearly exhibited precocity; in eight cells it underwent equational division; and in 33 cells its behavior could not be determined. In the course of this careful tabulation no chromosome other than the tiny rod was ever found to exhibit precocity, even in those cells in which this rod underwent equational division.

By way of summary of the cytological studies on spermatogenesis, then, it can be stated that for each of the X translocations, only one of the rearranged chromosomes is transmitted in duplicate through the sperm: in every case except OR T1 it is the chromosome which is made up of a proximal segment of X, including the X centromere and terminal heterochromatin. In OR T1, where the heterochromatin and centromere have become separated from each other, it is the rearranged chromosome composed of the X heterochromatin and a proximal segment of II which is transmitted in duplicate.

*Inheritance of the translocations through the male:* Once the chromosome complement included in the sperm nucleus had been determined for the various translocations, it was possible to anticipate the kinds of offspring that would be derived by breeding the males to normal females. This has been done in diagrammatic fashion in Figure 11 for all of the translocations except OR T1 and in Figure 10 for OR T1. In both diagrams the chromosome eliminations from the embryonic soma and germ line are based on the assumption that the translocation chromosome undergoing equational nondisjunction during spermatogenesis is likewise the chromosome to be eliminated during development. On this basis the daughters should be heterozygous (soma and germ line) for the translocation in question. That this is indeed the case was determined by genetic study and by cytological examination of the larval salivary and oögonial complements. The genetic tests consisted of phenotypic classification of the adult females and a similar classification of their progeny (see CROUSE 1943, 1960). Although the differentiation of sons is considered as a possibility in both diagrams, actually none was found; the male embryos failed to hatch out of the eggs. Their death at an early stage of embryonic development gives support to the correctness of the scheme of chromosome elimination followed in the diagrams. Presumably the

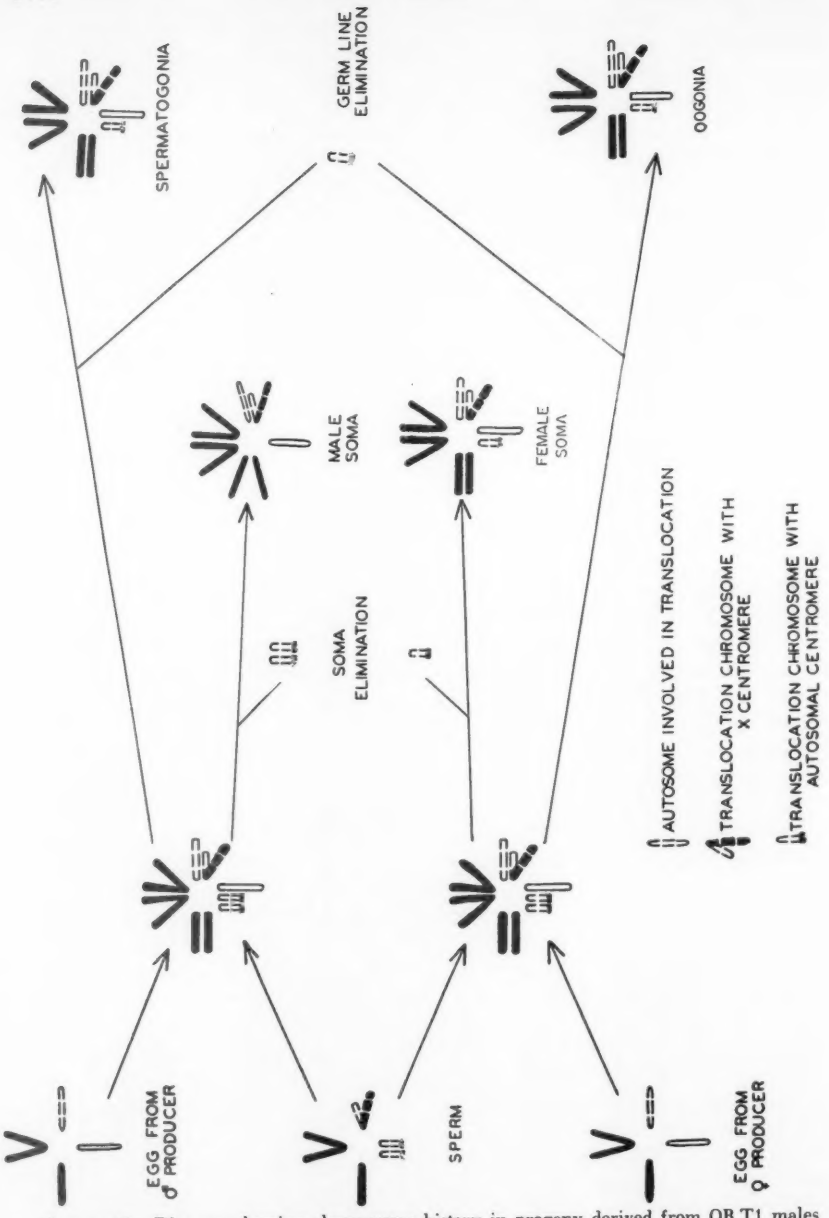



FIGURE 10.—Diagram showing chromosome history in progeny derived from OR T1 males bred to normal females.

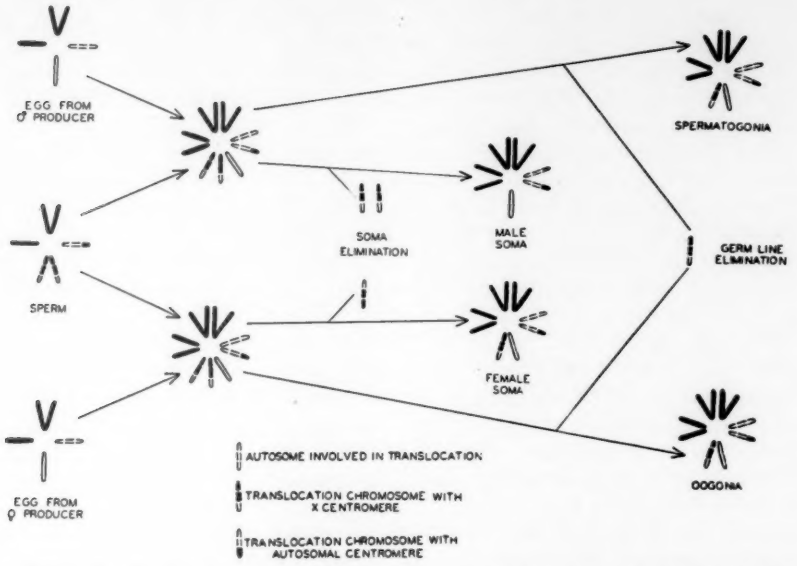



FIGURE 11.—Same as the diagram in Figure 10 except that it applies not to OR T1 but to the other eight translocations.

males die because of the unbalanced complement in their somatic nuclei: for each of the translocations the complement is deficient for a segment of autosome and duplicated for a segment of X, the X duplication in the case of OR T1 comprising all of the chromosome except the terminal mass of heterochromatin.

*Inheritance of the translocations through the female:* Meiosis in the Sciara female is perfectly orthodox and involves crossing over and the random assortment of homologues. Consequently, females heterozygous for an X translocation would be expected to form two classes of euploid eggs. That this is the case has already been established for both the Missouri (CROUSE 1943) and the Pennsylvania translocations (CROUSE 1960). It is true also of the Oak Ridge series (unpublished). In addition to the euploid eggs, aneuploid types arise through nondisjunction (CROUSE 1960). Aside from the phenomenon of nondisjunction, the X translocations are inherited in a regular, orthodox manner.

## DISCUSSION

The inheritance of sex chromosomes in Sciara and their pattern of behavior during development constitute a unique mechanism of sex determination and provide clear demonstration that the cells can distinguish one chromosome from another. This "recognition" results in the elimination of the chromosome or chromosomes from the cell and, thereby, in an altered genetic condition. The

translocation studies show clearly that it is not the whole X chromosome which is involved in the elaborate series of interrelated cytological events but only a very localized region, namely, the terminal (proximal) mass of heterochromatin. This region controls both equational nondisjunction in the secondary spermatocyte and chromosome elimination in the embryo; without it the sex chromosome behaves like an autosome, but with it the autosome behaves like X.

That the controlling region proves to be the heterochromatin which normally lies adjacent to the X centromere is, in the last analysis, probably not surprising. In view of the Sciara data one might have chosen the X centromere as the most plausible controlling element. First, the dramatic chromosome unorthodoxies in Sciara are clearly unrelated to the genic make-up of the chromosomes: a chromosome which passes through the male germ line acquires an "imprint" which will result in behavior exactly opposite to the "imprint" conferred on the same chromosome by the female germ line. In other words, the "imprint" a chromosome bears is unrelated to the genic constitution of the chromosome and is determined only by the sex of the germ line through which the chromosome has been inherited. Second, centric activity is obviously involved in equational nondisjunction, in chromosome elimination at anaphase (embryonic soma), and probably it is involved also in the prochromosome elimination from the germ cells at resting stage. That the controlling element on the X is not the centromere but an element which lies in the adjacent heterochromatin is in line with and gives support to the view advocated by NOVITSKI (1955) and by LINDSLEY and NOVITSKI (1958), that the kinetic activity of centromeres in Drosophila (as measured in anaphase bridge experiments) is to be attributed to the constitution of the heterochromatin immediately adjacent to the centromeres. That the heterochromatin of the Sciara X, upon translocation (OR T1) to the proximal segment of chromosome II, can control the behavior of the rearranged chromosome is indeed very interesting. Not only is the centromere considerably removed (opposite end of the rearranged chromosome) from the translocated heterochromatin, but it is a non-homologous centromere, the one derived from chromosome II. This result supports the view advocated by LINDSLEY and NOVITSKI (1958), that the function of the centromere is constant from chromosome to chromosome.

From the translocation studies it appears that a balanced chromosome complement is not essential in the early stages of Sciara development. The zygote can be either hypoploid or aneuploid and still give rise to an adult organism, provided a euploid XX or X0 somatic complement is acquired at the time of elimination. Thus zygotes which, as a result of nondisjunction, receive no sex chromosome from the egg nucleus, develop under the influence of two sex chromosomes (paternal) instead of three and become exceptional (patroclinous) males among the progeny of female-producing mothers (CROUSE 1960). And in the case of each X translocation taken through the male germ line, daughters are derived from aneuploid zygotes which carry an autosomal duplication and a deficiency on the X; in the case of OR T1 the deficiency includes all of the X chromosome except the terminal heterochromatin.

SUMMARY

Nine reciprocal translocations between chromosome X and the autosomes of *S. coprophila* have been studied in relation to their effects on spermatogenesis and chromosome elimination in the embryo. In every case one of the translocation chromosomes displayed behavior typical of the autosomes at the second spermatocyte division; the other exhibited precocity and thus underwent equational nondisjunction. The chromosome eliminated later from the embryonic soma and germ line was the translocation chromosome which had undergone equational nondisjunction during spermatogenesis. Precocity at the second spermatocyte division and elimination at the critical stages in the embryo were found to be under the control of the terminal heterochromatin which is located normally adjacent to the X centromere. When, by reciprocal translocation, this mass of heterochromatin comes to form the distal end of a rearranged chromosome which bears the proximal segment of II, it controls the behavior of this chromosome in spite of its wide separation from the centromere.

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# EFFECT OF AN ABNORMAL KNOB-CARRYING CHROMOSOME 10 ON PARAMUTATION OF $R^r$ IN MAIZE[1]

R. ALEXANDER BRINK AND WILLEM H. WEYERS

*University of Wisconsin, Madison, Wisconsin, and University of Natal, Pietermaritzburg, Natal, Union of South Africa*

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THE $R^r$ factor in maize, conditioning anthocyanin formation in seed and plant, is highly sensitive to alteration in pigment-producing action in heterozygotes with the stippled ($R^{st}$) or the marbled ($R^{mb}$) allele, when present in a structurally normal chromosome 10 (BRINK 1956; BRINK and WEYERS 1957). The $R^rr^gr^g$ kernels resulting from testcrosses of standard $R^rR^r$, $R^rr^r$, or $R^rr^g$ plants on $r^gr^g$ females (colorless aleurone, green plant) are darkly mottled, whereas the corresponding class of seeds from $r^gr^g$♀ × $R^rR^{st}$♂ and $r^gr^g$♀ × $R^rR^{mb}$♂ matings are weakly colored. The reduction in pigment-producing action that $R^r$ invariably undergoes in $R^rR^{st}$ and $R^rR^{mb}$ plants is heritable and has been attributed to a kind of genetic change at, or near, the $R^r$ locus termed paramutation (BRINK 1958).

Recent studies, the detailed results of which are unpublished, show that the sensitivity of $R^r$ to paramutation in heterozygotes with stippled is greatly altered by reciprocal translocations involving the long arm of chromosome 10, in which the *R* locus resides. The present report is concerned with a related investigation, namely, the effects on $R^r$ action following insertion of the factor into a chromosome 10 differing from the normal in possessing a unique terminal segment on the long arm that contains a large heterochromatic knob.

*Abnormal chromosome 10:* The knob-bearing, abnormal chromosome 10 in question, designated K10, has been found in several races of maize from Latin America and the southwestern United States and in a teosinte strain from Chapingo, Mexico (LONGLEY 1937, 1938; RHOADES 1952).

RHOADES (1942) observed that the proximal portion and a small distal portion of the distinctive terminal segment on the long arm of K10 are euchromatic. A conspicuous heterochromatic knob, about equal in length at pachytene to the sum of these euchromatic parts, lies between the latter. RHOADES found also that the proximal euchromatic part of K10 which is associated at pachytene with the distal one sixth of the normal chromosome 10, in K10/10 heterozygotes, differed in chromomere pattern. EMMERLING (1959) has identified three prominent chromomeres in this region that are not present in normal 10 and has observed that another, less conspicuous, chromomere lies at the base of the knob. Pairing between K10 and normal 10, in K10/10 heterozygotes, ends in the region between

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the most distal of the three prominent chromomeres and the chromomere close to the large heterochromatic knob. The segment distinguishing K10 gives no evidence of homology with any other part of the maize genome, nor with the B chromosomes occurring as supernumeraries in some maize plants. Thus, there is no clue to the origin of the structure.

A remarkable series of functional effects of abnormal chromosome 10 have been disclosed by the investigations of RHOADES, his students and associates, and of LONGLEY. About 70 percent of the functional megaspores formed by K10/10 heterozygotes carry the abnormal chromosome 10. RHOADES (1942) found that the deviation from 50 percent in such plants is a consequence of the formation of supernumerary spindle fibers along the chromosome and preferential assortment of K10 to the basal cell at megasporogenesis. Occurrence of the extra chromosome fibers is limited to the two meiotic divisions. Transmission of K10 through the pollen is subnormal. RHOADES (1942) and EMMERLING (1959) report frequencies between 40 and 45 percent in K10/10 heterozygotes.

LONGLEY (1945) observed that abnormal chromosome 10 promoted preferential segregation of other chromosome pairs if one homologue was knobbed and the other was knobless. This result has been confirmed by KIKUDOME (1959) who has shown also that in K10/10 plants the degree of preferential assortment of a knobbed chromosome 9 over a knobless mate is a function of chromosome 9 knob size. KIKUDOME found that such differential assortment of knobbed chromosome 9 did not occur in plants lacking K10.

Studies by EMMERLING (1959) show that the segment compromising the large heterochromatic knob and the euchromatic region distal to the knob has a major effect on preferential segregation. A modified abnormal chromosome 10, termed K°10, which lacks the knob and the distal segment, gave 52.7 percent transmission through the female gametophyte in K°10/10 plants in contrast with about 70 percent for K10 in K10/10 individuals. Neocentromeres are formed by K°10 plants, although their activity appears to be comparatively weak. The K°10 chromosome was found to disturb transmission of a knob-carrying chromosome 9, but at a greatly reduced level, as compared with abnormal chromosome 10.

Three readily usable marker genes are known in the long arm of chromosome 10: $g_1$ (golden plant), $R$ (colored aleurone), and $sr^s$ (striate-2). The order of these factors relative to the centromere, and the amounts of crossing over observed between them are $g_1$ 14 $R$ 35 $sr^s$ (KIKUDOME 1959). It was observed by RHOADES (1942) that in $G_1rK/g_1R$ plants, the amount of recombination between $g_1$ and $R$ was not affected by abnormal chromosome 10. The knob was shown to be distal to $R$ and to give only one-two percent recombination with it. RHOADES further observed that when crossing over occurred between $R$ and K the exchanges were proximal to the entire segment whereby K10 is distinguished from a normal chromosome 10. KIKUDOME (1959) confirmed this point and has shown also that in K10/10 heterozygotes the amount of crossing over between $R$ and $sr^s$ is reduced from about 35 percent to approximately 0.9 percent. These data, and the morphological evidence mentioned earlier, point to the conclusion

that, structurally at least, the proximal portion of the distinctive segment in abnormal 10 which corresponds in position at pachytene with the distal one sixth of normal chromosome 10, is not homologous with the latter. As pointed out previously, the remainder of the K10 differential segment appears to have no counterpart in the maize genome.

Plants homozygous for abnormal chromosome 10 are viable and fertile. Substitution in the genome of K10 for a standard chromosome 10, therefore, does not involve loss of any vital genes. Gross phenotypic differences between otherwise comparable K10/K10, K10/10 and 10/10 individuals have not previously been reported. The writers have observed, however, that in the genetic background of the W22 inbred strain K10/K10, but not K10/10, plants show the slightly zigzag stem condition characteristic of this line in an exaggerated degree, and ear weight is reduced to about 60 percent of the normal value.

## MATERIALS AND METHODS

A K10/K10 stock, homozygous for colorless aleurone ($r^r r^r$), kindly provided by PROFESSOR M. M. RHOADES, was outcrossed to our standard W22 $R^r R^r$ inbred line. The $R^r/r^r$K hybrid plants were pollinated with W22 $r^r r^r$. The approximately 30 percent of colored kernels resulting from this mating were planted in a detasselling plot, in which the W22 $r^r r^r$ stock was again used as the staminate parent. Of the 672 $R^r/r^r$ plants scored by inspection at harvest, 657 bore ears carrying a marked excess of colorless kernels and so were presumed to be $R^r/r^r$K noncrossovers, whereas 15 plants gave a marked excess of colored kernels and so were adjudged to be $R^r$K/$r^r$ crossovers. This is a recombination percentage between $R^r$ and K of 2.2. One of the 15 crossover plants became the progenitor of the W22 $R^r$K/$r^r$ stock culture which was drawn upon for the present experiments after two additional matings of $R^r$K/$r^r$ plants to the W22 $r^r r^r$ inbred line had been made.

The aleurone pigment-producing action of an *R* allele is best measured in single dose in kernels possessing *R r r* endosperms. Such seeds are obtained routinely by introducing *R* through the pollen in matings on *r r* (colorless aleurone) females. The resulting endosperm is triploid, as is normal in Angiosperms. The pistillate strains so employed in the present study were the W23 and W22 inbred strains, carrying the $r^g$ allele (colorless aleurone, green plant).

The testcross kernels were scored for pigmentation by matching against a set of standard seeds varying progressively from colorless (class 1) through five grades of mottling to self color (class 7).

## EXPERIMENTAL RESULTS

*Pigment-producing potential of* $R^r$*K and* $R^r$ *gametes:* The data assembled in Table 1 show that, on the average, the colored aleurone seeds resulting from $r^g r^g$ ♀ × $R^r$K/$r^g$ ♂ matings were somewhat darker than those from otherwise comparable $r^g r^g$ ♀ × $R^r r^g$ ♂ testcrosses. The difference, however, is only .42 of a class interval and is too small to be considered statistically significant. The F

TABLE 1

*Aleurone color scores of* $R^rr^gr^g$ *kernels following testcrosses on W23* $r^gr^g$ *females of W22* $R^r/r^g$ *and W22* $R^rK/r^g$ *plants*

| Family | $R^r/r^g$ male No. plants tested | Color score | Family | $R^rK/r^g$ male No. plants tested | Color score |
|---|---|---|---|---|---|
| | | (1) | | | (2) |
| 45–836 | 5 | 5.42 | 45–838 | 5 | 5.87 |
| –837 | 5 | 6.04 | –839 | 5 | 5.95 |
| –840 | 6 | 5.32 | –842 | 7 | 5.84 |
| –841 | 4 | 5.54 | –843 | 6 | 6.34 |
| Mean | 20 | 5.58 | Mean | 23 | 6.00 |

value, with one degree of freedom, is 4.62, whereas F .05 = 5.99 and F .10 = 3.78. It is evident that comparative tests on a larger scale than the present would be necessary to determine with confidence whether the aleurone pigment-producing potential of $R^rK$ gametes is actually slightly higher than that of $R^r$ gametes. It is clear from the available data, however, that if a real difference exists, it is small.

*Effect of abnormal chromosome 10 on paramutability of* $R^r$ *in heterozygotes with the marbled allele, and with self-colored mutants from marbled:* The $R^r$ allele decreases sharply in sensitivity to paramutation when carried by abnormal chromosome 10 in heterozygotes with the marbled factor on a normal chromosome ($R^rK/R^{mb}$). Data bearing on this relation are summarized in Table 2.

The pistillate parents used in all the testcrosses involving the $R^{mb}$ allele, and the two self-colored mutants from marbled, referred to later, were from the W22 $r^gr^g$ stock. The data obtained in this group of experiments (Tables 2 and 3), therefore, are not directly comparable with those presented elsewhere in the report, which are based upon matings with W23 $r^gr^g$, an unrelated inbred line. The staminate parents used in the testcrosses, however, were of the W22 strain throughout.

TABLE 2

*Aleurone color scores of* $R^rr^gr^g$ *testcross kernels from matings on W22* $r^gr^g$ *females of W22* $R^rR^{mb}$ *and W22* $R^rK/R^{mb}$ *plants*

| $r^gr^g♀ \times R^rR^{mb}♂$ (control) | | $r^gr^g♀ \times R^rK/R^{mb}♂$ | |
|---|---|---|---|
| Staminate parent | Mean score | Staminate parent | Mean score |
| W528C–1 | 5.12 | W525–1 | 6.45 |
| –2 | 4.55 | –2 | 6.64 |
| –3 | 4.62 | –3 | 6.36 |
| –4 | 4.81 | –4 | 6.71 |
| –5 | 4.32 | –5 | 6.70 |
| | | –6 | 6.51 |
| | | –7 | 6.68 |
| Mean | 4.68 | | 6.58 |

The marbled allele is unstable and varies from one subline to another, within the inbred W22 stock, with respect to both aleurone spotting pattern and paramutagenic competence. The effects of this instability were minimized in the present experiments by using an $R^{mb}$ factor from a common source in any given comparative test.

Testcrosses on W22 $r^g r^g$ females of seven $R^r$K/$R^{mb}$ plants, in family W525, yielded colored kernels with an average pigmentation score of 6.58, on the 1–7

TABLE 3

*Aleurone color scores of* $R^r r^g r^g$ *testcross kernels from matings on W22* $r^g r^g$ *females of W22* $R^r/R^{scm1}$ *and* $R^r K/R^{scm2}$ *plants.* $R^{scm1}$ *and* $R^{scm2}$ *are self-colored mutants from marbled*

| $R^{scm}$ allele | $r^g r^g$♀ × $R^r/R^{scm}$♂ (control) | | | $r^g r^g$♀ × $R^r$K/$R^{scm}$♂ | | |
|---|---|---|---|---|---|---|
| | Family | No. plants tested | Mean score | Family | No. plants tested | Mean score |
| $R^{scm1}$ | W618a | 4 | 3.92 | W618b | 6 | 6.20 |
| $R^{scm2}$ | W614 | 5 | 3.86 | W621 | 7 | 6.39 |
| $R^{scm2}$ | W615 | 6 | 2.80 | W624 | 5 | 5.43 |
| $R^{scm2}$ | W627 | 5 | 3.47 | W623 | 7 | 6.34 |
| | Mean | 20 | 3.51 | Mean | 25 | 6.09 |

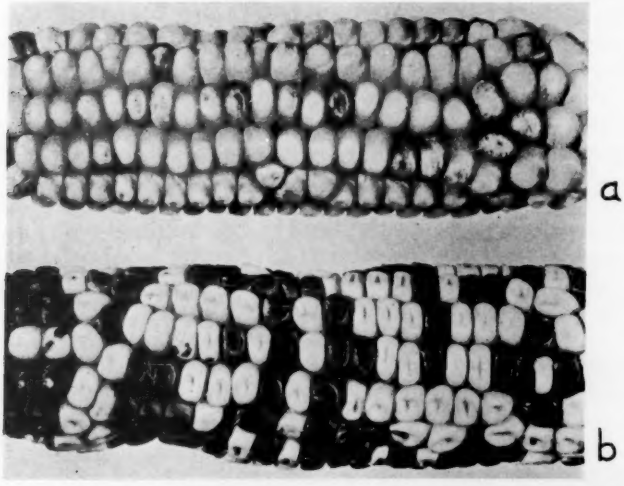


FIGURE 1.—(a): Ear from a W22 $r^g r^g$ ♀ × $R^r R^{mb}$ ♂ mating. The $R^{r:mb} r^g r^g$ kernels are weakly pigmented, and the $R^{mb}/r^g/r^g$ kernels are colorless, or nearly so. (b): Ear from a W22 $r^g r^g$ × $R^r$K/$R^{mb}$ mating. The $R^r$K/$r^g/r^g$ kernels are darkly pigmented, and the marbled kernels are colorless or spotted. Note that the level of aleurone pigmentation in the $R^r$K/$r^g/r^g$ kernels is much higher than that in their $R^{r:mb}/r^g/r^g$ counterparts, showing that $R^r$ when carried in abnormal chromosome 10 is comparatively insensitive to paramutation in heterozygotes with this marbled allele.

scale. The five control testcrosses, involving $R^r/R^{mb}$ individuals carrying $R^{mb}$ from the same parent plant and $R^r$ on a structurally normal chromosome 10, gave a corresponding score of 4.68. The difference, 1.90, is very highly significant statistically ($t = 14.8$, $P = < .001$). Representative ears from these two classes of testcrosses are illustrated in Figure 1. The $R^{mb}$ allele involved in this case is weakly paramutagenic, as evidenced by the fact that the mean aleurone color score for the control kernels, from the $R^rR^{mb}$ matings, is higher than usual for such testcrosses, many of which have been made in other experiments with marbled. The important fact, however, is that this weakly paramutagenic $R^{mb}$ allele has little or no effect on the pigment-producing action of the standard $R^r$ factor in $R^r\text{K}/R^{mb}$ plants. Evidently $R^r$, when carried by abnormal chromosome 10, is quite insensitive to paramutation in heterozygotes with this particular $R^{mb}$ factor.

The effect on paramutability of $R^r$ when $R^r$ is borne by chromosome 10 in heterozygotes with two self-colored mutants from marbled, designated $R^{scm1}$ and $R^{scm2}$, is shown by the data in Table 3. It will be noted that the mean scores for aleurone color from the control matings, involving $R^r/R^{scm}$ plants, are low, showing that the $R^{scm1}$ and $R^{scm2}$ mutants from $R^{mb}$ are strongly paramutagenic in plants normal for chromosome 10. $R^r$, when carried by a K10 chromosome, however, is relatively insensitive to the paramutagenic action of these factors. The control family in the case of $R^{scm1}$ gave a mean aleurone color score of 3.92, whereas this value for the corresponding $R^r\text{K}/R^{scm1}$ individuals was 6.20. The difference in score, 2.28, is highly significant statistically ($t = 14.6$; $P < .01$). Similar, highly significant, differences ($P < .01$) were obtained in each of the three paired comparisons involving $R^{scm2}$. It is apparent from this group of data that the two self-colored mutants from marbled, although strongly paramutagenic, as shown by the results of the control tests, have little effect on the pigment-producing potential of $R^r$ when the latter factor is carried by abnormal chromosome 10 in $R^r\text{K}/R^{scm}$ plants.

*Tests involving abnormal chromosome 10 and the stippled* ($R^{st}$) *allele:* The $R^r$ factor, when inserted in abnormal chromosome 10, decreases in sensitivity to the paramutagenic action of $R^{st}$ also, as shown by the data in Table 4. This conclusion is based upon the results of tests of the pigment-producing potentials of $R^r$ and $R^r\text{K}$ gametes from $R^r/R^{st}\text{K}$ and $R^r\text{K}/R^{st}\text{K}$ plants, that is to say, from individuals in which the stippled allele, itself, also was carried on abnormal chromosome 10. $R^r\text{K}/R^{st}$ plants were not included in the experiment. The first question presented in considering these data, therefore, is whether the paramutagenic action of stippled is affected by inserting the factor into abnormal chromosome 10.

*Effect of K10 on the paramutagenic action of* $R^{st}$: Columns (2) and (4) show the aleurone color scores of the $R^rr^gr^g$ kernels resulting from the pollination of $r^gr^g$ females by $R^rR^{st}$ and $R^r/R^{st}\text{K}$ plants, respectively. The mean values for the $r^gr^g\,♀ \times R^rR^{st}\,♂$ matings (Families 45–836 and 45–837) are 2.59 and 2.48, and thus correspond closely. The two families (45–840 and 45–841) representing the $r^gr^g\,♀ \times R^r/R^{st}\text{K}$ testcrosses gave average scores of 2.90 and 3.40. The difference between families in this latter case is considerable, but falls below the level of

TABLE 4

*Aleurone color scores of* $R^rr^gr^g$ *kernels following testcrosses on W23* $r^gr^g$ *females of W22* $R^r/R^{st}$, $R^r/R^{st}K$, *and* $R^rK/R^{st}K$ *plants*

| $R^r/R^{st}$ males | | $R^r/R^{st}$K males | | $R^r$K/$R^{st}$K males | | $R^r$K/$r^g$ males | |
|---|---|---|---|---|---|---|---|
| Male parent | Color score | Male parent | Color score | Male parent | Color score | Male parent | Color score |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
| 45–836–4 | 2.68 | 45–840–2 | 3.13 | 45–842–4 | 4.93 | 45–842–1 | 6.28 |
| –6 | 2.50 | –4 | 2.97 | –7 | 4.87 | –6 | 5.97 |
| –13 | 2.57 | –5 | 3.00 | –14 | 3.82 | –8 | 5.47 |
| –15 | 2.43 | –9 | 2.97 | –18 | 5.53 | –10 | 6.10 |
| –17 | 2.75 | –16 | 2.43 | –19 | 4.27 | –23 | 5.87 |
| | | | | –25 | 4.63 | –26 | 5.40 |
| | | | | –29 | 4.48 | –35 | 5.78 |
| Mean | 2.59 | Mean | 2.90 | Mean | 4.65 | Mean | 5.84 |
| 45–837–2 | 2.18 | 45–841–8 | 3.92 | 45–843–2 | 6.62 | 45–843–1 | 6.62 |
| –6 | 2.42 | –15 | 3.55 | –7 | 6.33 | –3 | 6.85 |
| –11 | 2.23 | –39 | 3.03 | –8 | 6.20 | –6 | 5.87 |
| –13 | 3.40 | –44 | 3.12 | –19 | 6.58 | –10 | 6.78 |
| –15 | 2.17 | | | –20 | 5.73 | –11 | 6.00 |
| | | | | –22 | 6.42 | –23 | 5.90 |
| Mean | 2.48 | Mean | 3.40 | Mean | 6.31 | Mean | 6.34 |
| Weighted group mean | 2.53 | Weighted group mean | 3.12 | Weighted group mean | 5.42 | Weighted group mean | 6.07 |

statistical significance ($d = .50$; $LSD_{.05} = .55$). The weighted mean scores for $R^rr^gr^g$ kernels from the two classes of matings are 2.53 and 3.12, and the difference, .59, is in the direction of a reduced paramutagenic action of the $R^{st}$ allele when carried in abnormal chromosome 10. This difference, however, is not significant statistically ($d = .59$; $LSD_{.05} = 2.05$). The available data, however, fall short of being conclusive. The possibility is to be borne in mind, therefore, that if a larger scale test of the $R^r$ gametes from $R^rR^{st}$ and $R^r/R^{st}$K plants had been made, a significant depressing effect of abnormal chromosome 10 on the paramutagenic action of the stippled allele might have been disclosed. However this may be, it is clear that the paramutagenic action of $R^{st}$ in an abnormal chromosome 10 is still strong so that the $R^{st}$K combination may be effectively used, as shown in the following section, in testing whether the paramutability of $R^r$ is changed when $R^r$ is inserted into K10.

*Paramutation in* $R^r/R^{st}K$ *and* $R^rK/R^{st}K$ *heterozygotes:* The aleurone color scores for the $R^rr^gr^g$ kernels from $r^gr^g$♀ × $R^r/R^{st}$K♂ matings are summarized in column (4) in Table 4. As noted in the preceding paragraph, the mean values for the two families used, 45–840 and 45–841, are 2.90 and 3.40, respectively, and the weighted average is 3.12. The corresponding means for the testcrosses involving $R^rK/R^{st}K$ plants, on the other hand, for Families 45–842 and 45–843, as given in column (6), are 4.65 and 6.31. The difference between the family means

is highly significant statistically ($d = 1.67$; $LSD_{.01} = 1.03$). The reason for the heterogeneity is obscure because the two families are of the same, closely inbred, W22 stock. Possibly the regularity of expression of paramutation in the presence of abnormal chromosome 10 is subject to the action of factors not operative in normal plants.

The existence of heterogeneity in the data from the two $r^g r^g$ ♀ × $R^r$K/$R^{st}$K testcross families makes a comparison with the results from the $r^g r^g$ ♀ × $R^r/R^{st}$K ♂ matings by conventional statistical procedures of doubtful value. The difference between the weighted group means is 2.30 units, the darker kernels being those in which the $R^r$ allele is borne by abnormal chromosome 10. The magnitude of the difference, and the fact that the distributions of the scores for the respective $R^r r^g r^g$ and $R^r$(K)$r^g r^g$ ear families overlap in one case only, suggest that presence in an abnormal chromosome 10 renders $R^r$ comparatively insensitive to the paramutagenic action of the $R^{st}$ allele.

Additional evidence that the sensitivity of $R^r$ to paramutation in $R^{st}$ heterozygotes is low when $R^r$ is borne by abnormal chromosome 10 is afforded by comparison of the results of testcrosses on $r^g r^g$ females of $R^r$K/$R^{st}$K and $R^r$K/$r^g$ plants, as summarized in columns (6) and (8) of Table 4. The two classes of plants in each of these families are sibs, resulting from $R^r$K/$R^r$K × $R^{st}$K/$r^g$ matings. The effect of substituting $R^{st}$K for $r^g$ in the heterozygotes with $R^r$K, in Family 45–842, is to reduce the mean color score of the $R^r r^g r^g$ testcross kernels from 5.84 to 4.65. The latter value is still 1.25 units above the level of the higher (Family 45–841) of the two mean values observed in the tests with $R^r/R^{st}$K plants. This result, if taken by itself, indicates that $R^r$ in the $R^r$K combination is only moderately paramutable as compared with $R^r$ in a normal chromosome 10. Family 45–843 affords more conclusive data. The mean color score, following $r^g r^g$ ♀ × $R^r$K/$r^g$ ♂ testcrosses, was 6.34, and the corresponding value for the mating involving $R^r$K/$R^{st}$K sibs was 6.31. In this family, therefore, the $R^r$ allele borne by the K10 chromosome showed no sensitivity to paramutation.

*Sensitivity of* $R^r$ *to paramutation following extraction from abnormal chromosome 10:* The $R^r$ allele has been recovered in a normal chromosome from an abnormal chromosome 10, by crossing over in an $R^r$K10/$r^r$10 plant, and then tested for paramutability, in a single case only. Since the amount of recombination between $R^r$ and K is of the order of one percent, and presence in a plant of an abnormal 10 chromosome is most conveniently detected by distortion of the ratios of the linked $R$ factor on segregating ears, special measures are necessary to obtain an adequate sample of such crossovers in a combination suitable for testing directly the effect of the exchange on paramutability of $R^r$. The results now at hand are assembled in Table 5.

Families W522 and W523 were closely comparable in that the $R^r$-carrying abnormal chromosome 10 and $R^r$-carrying normal chromosome 10 were derived from sister plants, and the $R^{mb}$ allele in both cases came from the same individual. As shown in Table 5, the mean aleurone color score in the $R^r$K/$R^{mb}$ testcrosses was 5.10, whereas this value decreased to 3.46 in the testcrosses involving $R^r R^{mb}$ plants carrying $R^r$ extracted from abnormal chromosome 10. The difference in

average color grade is 1.64, and is highly significant statistically ($t = 5.2$; $P = < .001$). So far, therefore, as may be ascertained from this single instance, an $R^r$ allele returned from an abnormal chromosome 10 to a normal chromosome by crossing over regains sensitivity to paramutation. The regularity with which this result occurs and whether such recovered $R^r$ alleles are sensitive to paramutation in the same degree as their counterparts from $R^rR^r$ stock cultures are unanswered questions.

## DISCUSSION

The data presented show that the sensitivity of $R^r$ to paramutation in heterozygotes with the stippled or marbled alleles, or with paramutagenic self-colored mutants from marbled, is greatly decreased when $R^r$ is inserted into abnormal chromosome 10.

This result is not associated with the meiotic abnormalities which K10 incites in K10/10 heterozygotes. Unpublished data prove that paramutation of $R^r$, in heterozygotes of appropriate kinds, occurs in somatic cells during development of the plant. Furthermore, an experiment designed to determine whether conjugation at zygotene in meiosis is a condition of paramutation gave a negative result (Brink 1959). It would not be expected, therefore, that meiotic disturbances, as such, would affect $R^r$ paramutability; and the data in Tables 2, 3, and 4 show that this is indeed the case. The $R^r$ allele in $R^r$K/$R^{st}$K plants is comparatively insensitive to paramutation, even though, as Rhoades (1952) observed, pairing and distribution of abnormal chromosome 10, is regular in such individuals. The factor, likewise, was found to be relatively insensitive to change in $R^r$K/$R^{mb}$ and $R^r$K/$R^{scm}$ plants, in which chromosome 10 assorts preferentially. It may be concluded that the effect of abnormal chromosome 10 on sensitivity of $R^r$ to paramutation is a somatic phenomenon.

The terminal appendage distinguishing abnormal chromosome 10 has been shown by previous investigators to be a heterogeneous structure. The single most conspicuous component is a large heterochromatic knob. Proximal and distal to

TABLE 5

*Aleurone color scores of* $R^rr^gr^g$ *testcross kernels from matings on W22* $r^gr^g$ *females of plants carrying an* $R^r$ *allele (a) in an abnormal chromosome 10 and (b) extracted from an abnormal 10 chromosome 10 by crossing over*

| $r^gr^g$♀ × $R^r$K/$R^{mb}$♂ | | $r^gr^g$♀ × crossover $R^r/R^{mb}$♂ | |
|---|---|---|---|
| Male parent | Color score | Male parent | Color score |
| W522–1 | 4.81 | W523–1 | 3.00 |
| –2 | 5.20 | –2 | 4.27 |
| –3 | 4.65 | –3 | 3.48 |
| –4 | 5.53 | –4 | 3.56 |
| –5 | 5.31 | –5 | 3.89 |
| | | –6 | 2.56 |
| Mean | 5.10 | Mean | 3.46 |

this body, however, are seemingly euchromatic regions. The present data do not discriminate between these different parts with respect to source of the K10 effect on paramutability of $R^r$. By analogy with variegating position effects in *Drosophila melanogaster* (LEWIS 1950), which involve euchromatic-heterochromatic transpositions, the heterochromatic knob would appear to be the most likely basis of the phenomenon. The fact, as mentioned earlier, that certain reciprocal translocations involving the long arm of chromosome 10 and not known to alter the position of $R^r$ relative to a major heterochromatic element, nevertheless do change the paramutability of $R^r$ shows, however, that the analogy may not hold. On the other hand, the present evidence does not exclude the heterochromatic knob in abnormal chromosome 10 as the source of the change in sensitivity of $R^r$ to paramutation.

$R^r$, on being returned to a normal chromosome from an abnormal chromosome 10, by crossing over, in the single instance tested, was found to be sensitive to paramutation at about the standard level. This result suggests that the change in $R^r$ action in a K10 chromosome is a position effect of the conventional kind (LEWIS 1950). This may prove to be a fact, but there are reasons for not accepting such a conclusion as final at present.

WEYERS (1959) found that the marbled allele varies appreciably in paramutagenic action from one subline of the W22 inbred stock to another, and seemingly cannot be stabilized in this repect. This circumstance limits accordingly the significance of the result of any single test for degree of paramutability of $R^r$ in an $R^rR^{mb}$ heterozygote. It is to be noted also that, in the present instance, an $R^rR^{mb}$ heterozygote carrying the $R^{mb}$ allele in question and an $R^r$ factor from an $R^rR^r$ stock culture was not available as a direct control. The fact established by the results summarized in Table 5 is that an $R^r$ allele extracted from abnormal chromosome 10 shows a considerable increase in sensitivity to paramutation. Whether the change is actually back to the level characteristic of standard $R^r$, or is a value above or below this level, is a question that can be answered only when a larger sample of $R^r$ crossovers is tested directly against standard $R^r$ controls.

There is a second reason for reserving judgment on the absence of a carry-over effect, with respect to paramutability of $R^r$, following extraction of the factor from abnormal chromosome 10. It has been observed that certain other structural changes (reciprocal translocations) involving, in each case, a rearrangement either proximal or distal to $R^r$ in the long arm of chromosome 10, incite changes at or near the $R$ locus, affecting paramutability of $R^r$, that persist strongly following return of the factor from a structurally altered to a normal chromosome. These results, which have not been published except in abstract form (BRINK, BLACKWOOD, and NOTANI 1960) suggest that the functional properties of $R^r$ are conditioned, in some unknown way, by integrity of the long arm of chromosome 10 in which the $R$ locus is situated. Any structural derangement of this chromosome region, therefore, is under suspicion as affecting the properties of $R^r$ in a persistent way, unless proved to the contrary. The presently available data do not disclose a persistent effect on $R^r$ action of abnormal chromosome 10, but they are not adequate to exclude such an influence.

SUMMARY

The $R^r$ factor conditioning aleurone and plant color in maize, which had been shown previously to be highly paramutable in heterozygotes with the stippled or marbled allele in cytologically normal plants, was found to be relatively insensitive to paramutation when inserted into an abnormal chromosome 10 containing a large heterochromatic knob (K10), and then similarly tested. Abnormal chromosome 10 is present in some maize strains from Latin America and the southwestern United States and has been shown by others to cause certain striking meiotic irregularities in heterozygous plants, leading to preferential distribution of the knob-carrying chromosome to the functional megaspore. Paramutation is known to occur in somatic cells and so, as expected, was found not to be influenced by the meiotic peculiarities of K10 individuals. $R^r$, on being returned to a normal chromosome from abnormal chromosome 10 by crossing over, in the single case tested, proved to be again relatively sensitive to paramutation in heterozygotes with the marbled allele.

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Funk Brothers Seed Co.
Bloomington, Ill.—1950

Ghostley's Poultry Farm
Anoka, Minnesota—1956

Greenwood Seed Company
Greenwood Farms
Thomasville, Georgia—1959

Kimber Farms, Inc.
Niles, California—1953

Moews Seed Co.
Granville, Ill.—1949

Nicholas Turkey Breeding Farms
Sonoma, California—1958

Nichols Poultry Farm
Kingston, N. H.—1950

Northrup King & Co.
Minneapolis 15, Minn.—1957

Pfister Associated Growers, Inc.
Aurora, Ill.—1957

Pioneer Hi-Bred Corn Co.
Johnston, Iowa—1949

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